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(54) Title: SEQUENTIAL TARGETING OF TUMOR SITES WITH OLIGONUCLEOTIDE CONJUGATES OF ANTIBODY AND COMPLEMENTARY OLIGONUCLEOTIDE CONJUGATES OF CHELATED RADIONUCLIDE (57) Abstract <p>The present invention is directed to a non-radioactive targeting immunoreagent that comprises oligonucleotides comprised of non-self-associating oligonucleotide sequences, and one or more linking groups and a radioactive targeting immunoreagent that comprises an oligonucleotide comprised of an oligonucleotide sequence that is complementary in sequence to and capable of hybridization with one or more fragments of a non-self-associating oligonucleotide sequence, one or more chelating agents, one or more linking groups and one or more radionuclides. The present invention is also directed to pharmaceutical compositions comprising one or more of the above-described immunoreagents and a pharmaceutically acceptable carrier. The present invention is further directed to methods for treating and imaging disease sites in patients.</p>		

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SEQUENTIAL TARGETING OF TUMOR SITES WITH OLIGONUCLEOTIDE
CONJUGATES OF ANTIBODY AND COMPLEMENTARY
OLIGONUCLEOTIDE CONJUGATES OF CHELATED RADIONUCLIDE

5 Field of the Invention

 This invention relates to sequential targeting and
delivery of radioactive immunoreagent compositions which
find particular utility in therapeutic and diagnostic
imaging methods. This invention also relates to novel
10 methods for the attachment of oligonucleotides,
complementary oligonucleotides, and chelates, to
proteins, and to bifurcated tumor targeting and delivery
vectors for the treatment and diagnostic imaging of
tumors.

15

Background of the Invention

 The various currently available radiolabeled
immunoreactive proteins which are employed in diagnostic
imaging and targeted therapeutic applications suffer
20 from one or more of the following disadvantages:
1) toxicity;
2) destruction of the reagent due to rapid metabolism;
3) inadequate emission characteristics such as low
signal to noise ratio;
25 4) inefficient covalent bonding with protein for
conjugate preparation;
5) slow complexation with metals;
6) unstable metal complexation, e.g., with respect to
temperature, time or pH;
30 7) inability to form conjugates until after metal
complexation is accomplished;
8) inability to spectrophotometrically analyze the
radionuclide complex reagent;
9) inability to form complexes without activation
35 steps that degrade protein;

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- 10) prolonged plasma half-lives of radioactive immunoconjugates result in prolonged exposure of normal tissue to damaging effects of radiation;
- 11) slow clearance of radionuclide from the body; and
- 5 12) increasing the number of sites of incorporation of radionuclide or chelated radionuclide results in a reduction in the capacity of the radiolabeled immunoreactive protein to bind to its target.

10 It is an object of the present invention to overcome the aforementioned disadvantages of the currently available radiolabeled immunoreactive proteins.

Summary of the Invention

15 The present invention is directed to a non-radioactive targeting immunoreagent that comprises a tumor antigen recognizing moiety, one or more oligonucleotides comprised of non-self-associating oligonucleotide sequences, and one or more linking

20 groups.

The present invention is also directed to a radioactive targeting immunoreagent that comprises an oligonucleotide comprised of an oligonucleotide sequence that is complementary in sequence to and capable of

25 hybridization with one or more fragments of a non-self-associating oligonucleotide sequence, one or more chelating agents, one or more linking groups and one or more radionuclides.

The present invention is also directed to

30 pharmaceutical compositions comprising one or more of the above-described immunoreagents and a pharmaceutically acceptable carrier.

The present invention is further directed to methods for treating and imaging disease sites such as

35 tumor sites in a patient. Said methods comprise

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administration to the patient of an effective amount of the above-described non-radioactive targeting immunoreagent followed at an effective time interval by an effective amount of the above-described radioactive targeting immunoreagent.

The present invention provides many advantages compared to conventional targeting immunoreagents. For example, the non-radioactive targeting immunoreagent can accumulate at a tumor site in vivo while it is not accumulated at normal tissue sites.

The in vivo residence half life of the non-radioactive targeting immunoreagent is long enough to permit its accumulation at a tumor site.

The in vivo residence half life of the radioactive targeting reagent is shorter than the residence half life of the non-radioactive targeting immunoreagent.

The portion of the radioactive targeting reagent that does not hybridize to tumor associated non-radioactive targeting reagent is rapidly cleared from the patient.

With respect to the same degree of modification of a targeting immunoreagent by a directly labeled radionuclide or a chelate containing a radionuclide, an amplification of the number of radionuclides per site of modification per targeting immunoreagent can be obtained.

A segment of the complementary sequenced oligonucleotides of the non-radioactive targeting immunoreagent and a segment of the radioactive targeting reagent can hybridize in vitro and in vivo.

The complementary sequenced oligonucleotides of the non-radioactive targeting immunoreagent and the radioactive targeting reagent are stable to exonuclease activity.

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The complementary sequenced oligonucleotides of the non-radioactive targeting immunoreagent and the radioactive targeting reagent do not self hybridize.

5 The non-radioactive targeting immunoreagent and the radioactive targeting reagent can comprise a wide variety of spacing, linking, and chelating groups, oligonucleotide sequences and radionuclides.

10 The complementary sequenced oligonucleotides of the non-radioactive targeting immunoreagent can be comprised of oligonucleotide sequences which can be tandemly linked by spacing groups, wherein a segment of each oligonucleotide sequence can hybridize with a segment of the radioactive targeting reagent.

15 The complementary sequenced oligonucleotides of the non-radioactive targeting immunoreagent can be linked to an antibody by means of either a 5'- or a 3'-substituent such as a 5'-amine or 3'-amine.

20 Reagents are provided that have a specificity for tumors and a wide variety of compositions, size and molecular weight can be prepared in accordance with the present invention.

25 A particular advantage of the present invention is that oligonucleotide sequence lengths and spacing groups can be selected such that on hybridization of two complementary radioactive targeting reagent oligonucleotide sequences to a single oligonucleotide-containing strand comprised of adjacent, tandemly linked oligonucleotide sequences of a non-radioactive targeting immunoreagent, the proximal end groups of the sequences
30 of two such radioactive targeting moieties are orthogonal to each other because of their relative spacial configuration on the double stranded helix so formed.

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Other advantageous features of this invention will become readily apparent upon reference to the following description of the preferred embodiments.

5 Description of the Preferred Embodiments

 This invention describes various novel bioconjugates which possess utility in therapeutic and diagnostic imaging compositions and methods. This invention further describes novel methods of preparing
10 bioconjugates by the attachment of various oligonucleotide sequences to chelating agents, preferably terpyridine containing chelating agents, and to immunoreagents such as proteins, antibodies, and receptors.

15 In particular, this invention describes novel bioconjugates useful for sequential targeting and amplified delivery of novel radioactive immunoreagent compositions which find particular utility in therapeutic and diagnostic imaging compositions and
20 methods.

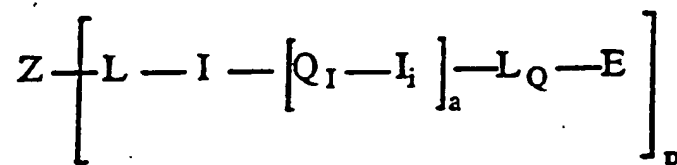
 More particularly, this invention describes the preparation and use of targeting immunoreagents that comprise a tumor antigen recognizing moiety, one or more oligonucleotides comprised of non self-associating
25 oligonucleotide sequences, and one or more linking groups. These targeting immunoreagents react with a radioactive sequential targeting reagent that comprises an oligonucleotide comprised of a oligonucleotide sequence that is complementary in sequence to and
30 capable of hybridization with one or more fragments of the said non self-associating oligonucleotide sequences, one or more chelating agents, one or more linking groups, and having one or more radionuclides associated therewith. Most preferably, the nucleotides are
35 deoxyribonucleotides.

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In a preferred embodiment, the above-described targeting immunoreagents form a compound that comprises moieties represented by the structure 1:

Structure 1

5



wherein:

10

Z comprises the residue of an immunoreactive protein;

L and L_Q are independently a chemical bond or a linking group;

15

I comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous contiguous sequences, the individual homologs of said family being comprised of from 12 to about 30 nucleotide units, and provided that contiguous sequences of six or more nucleotide units of said oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous nucleotide units anywhere in structure 1;

25

Q_I is a spacing group;

a is 0 or an integer from 1 to about 6;

I_i comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units, a contiguous sequence therein comprising a portion of I;

30

E is an end capping group; and

p is an integer from 1 to about 10.

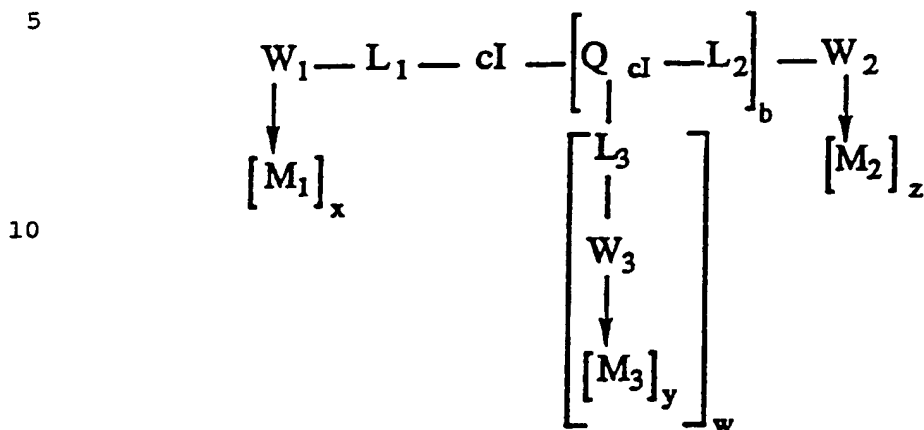
More preferably, a is an integer from 1 to about 6.

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In another preferred embodiment, the above-described targeting reagent is comprised of moieties represented by the structure 2:

Structure 2



wherein:

cI comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units wherein said contiguous sequence contains therein one or more members of a family of homologous contiguous sequences, the individual homologs of said family being comprised of from 12 to about 30 nucleotide units; where the nucleotide sequences of said members of said family of homologous contiguous sequences are complementary to the nucleotide sequences of members of the set of oligonucleotides in a coadministrable targeting immunoreagent and contiguous sequences of six or more nucleotide units of said complementary oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous nucleotide units anywhere in structure 2;

Q_{cI} is a spacing group;

L_1 , L_2 , and L_3 are independently a chemical bond or a linking group;

W_1 , W_2 , and W_3 are residues of chelating groups;

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M_1 , M_2 , and M_3 are comprised of elements with oxidation states equal to or greater than +1, and at least one of M_1 , M_2 , and M_3 is a radionuclide;

5 x , y , and z are independently zero or one provided that at least one of x , y , or z is one; and

w and b are independently zero or an integer from 1 to about 4.

 The term "residue" as used herein in context with a chemical entity, which chemical entity is comprised of,
10 for example, a chelating group, or a linking group, or a protein reactive group, or an immunoreactive group, or an immunoreactive protein, or an antibody, or an antibody fragment, or a cross-linking agent such as a heterobifunctional cross-linking agent, or an
15 oligonucleotide, or a spacing group, or an end capping group, is defined as that portion of said chemical entity which exclusively remains when one or more chemical bonds of said chemical entity when considered as an independent chemical entity, are altered,
20 modified, or replaced to comprise one or more covalent bonds to one or more other chemical entities. Thus, for example, in one aspect, a "residue of an oligonucleotide" in the context of, for example, I and I_i in Structure 1 or of cI in Structure 2 is comprised
25 of an oligonucleotide modified at least for divalent attachment to the residue of another chemical entity, i.e., the residue of said oligonucleotide is comprised of at least a divalent oligonucleotidyl sequence. In another aspect, for example, "the residue of a chelating
30 group" in the context of W_1 , W_2 , or W_3 of Structure 2 is comprised of a chelating group which is at least monovalently modified through attachment to the residue of another chemical entity such as, for example, to the residue of a linking group.

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In Structure 1 above, Z preferably is an antibody or antibody fragment which recognizes and is specific for a tumor associated antigen. In some embodiments, the above-described protein can contain an

5 immunoreactive group covalently bonded thereto through a chemical bond or a linking group derived from the residue of a protein reactive group and the residue of a reactive group on the protein. As used herein, the term "immunoreactive protein" which can be abbreviated by

10 "IRP" also includes an organic compound which is capable of covalently bonding to the protein and which is found in a living organism or is useful in the diagnosis, treatment or genetic engineering of cellular material or living organisms, and which has a capacity for

15 interaction with another component which may be found in biological fluids or associated with cells to be treated such as tumor cells.

Depending upon the intended use, the IRPs can be comprised of a wide variety of naturally occurring or

20 synthetically prepared materials, including, but not limited to, in part or in their entirety, enzymes, amino acids, peptides, polypeptides, proteins, lipoproteins, lipids, phospholipids, glycoproteins, hormones, drugs (for example digoxin, phenytoin, phenobarbital,

25 thyrozone, triiodothyronine, gentamicin, carbamazepine, and theophylline), steroids, vitamins, polysaccharides, viruses, protozoa, fungi, parasites, rickettsia, molds, and components thereof, blood components, tissue and organ components, pharmaceuticals, haptens, lectins,

30 toxins, nucleic acids (including oligonucleotides), antibodies, antigenic materials (including proteins and carbohydrates), avidin and derivatives thereof, biotin and derivatives thereof, and others known to one skilled in the art.

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Preferred IRPs are those which are comprised of the residue of a receptor molecule specific to the residue of a ligand of interest. Thus, a specific binding reaction involving the immunoreagent can be used for the targeting of a ligand of interest. Examples of such receptor-ligand combinations include, but are not limited to antibody-antigen, avidin-biotin, repressor (inducer) - promoter of operons and sugar-lectin complexes. Additionally, complementary nucleic acid sequences, i.e., a hybridized product of complementary strands, are also considered specific binding materials as the term is used herein. Useful IRPs are comprised of residues of substances which include (1) any substance which, when presented to an immunocompetent host, will result in the production of a specific antibody capable of binding with that substance, or (2) the antibody so produced, which participates in an antigen-antibody reaction. Thus, the IRP can be an antigenic material, an antibody, or an anti-antibody. Both monoclonal and polyclonal antibodies are useful. The antibodies can be whole molecules or various fragments thereof, as long as they contain at least one reactive site for reaction with the reactive groups on the precursors of the linking groups as described hereinbelow.

As used herein, the term "antibody fragment" is used to describe any material derived from antibodies but which retains the ability to recognize and bind to an antigen. Such fragments may, for example, be produced by means of:

- (i) Enzymatic digestion (e.g., Fab or Fab₂ fragments);
- (ii) Chemical means (e.g., the fragments produced by acid hydrolysis); or

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(iii) Products created by molecular biology of or genetic engineering of the antibody genes (e.g. single chains or Fv fragments).

5 In certain embodiments, the IRP can be comprised of an enzyme which has a reactive group for attachment to the precursors of the linking groups as described hereinbelow. Representative enzymes include, but are not limited to, aspartate aminotransaminase, alanine aminotransaminase, lactate dehydrogenase, creatine
10 phosphokinase, gamma glutamyl transferase, alkaline acid phosphatase, prostatic acid phosphatase, horseradish peroxidase and various esterases.

If desired, the IRP can be modified or chemically altered to provide reactive groups for attaching to the
15 residues of precursors of the linking groups or to oligonucleotides by techniques known to those skilled in the art. Such techniques include the use of linking moieties and chemical modification such as described in WO-A-89/02931 and WO-A-89/2932, which are directed to
20 modification of oligonucleotides, and U.S. Patent No. 4,719,182.

Two highly preferred uses for the compositions of this invention are for the diagnostic imaging of tumors and the radiological treatment of tumors. Preferred
25 immunological groups therefore include antibodies or antibody fragments such as, for example, Fab, Fab'2, and single chain Fv fragments to tumor-associated antigens. Specific non limiting examples include B72.3 and related antibodies (described in U.S. Patent Nos. 4,522,918 and
30 4,612,282) which recognize colorectal tumors, 9.2.27 and related anti-melanoma antibodies, D612 and related antibodies which recognize colorectal tumors, UJ13A and related antibodies which recognize small cell lung carcinomas, NRLU-10 and related antibodies which
35 recognize small cell lung carcinomas and colorectal

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tumors (Pan-Adenocarcinoma), 7E11C5 and related antibodies which recognize prostate tumors, CC49 and related antibodies which recognize colorectal tumors, TNT and related antibodies which recognize necrotic tissue, PR1A3 and related antibodies, which recognize colon carcinoma, ING-1 and related antibodies, which are described in International Patent Publication WO-A-90/02569, B174 and related antibodies which recognize squamous cell carcinomas, B43 and related antibodies which are reactive with certain lymphomas and leukemias, and anti-HLB and related monoclonal antibodies. An especially preferred antibody is ING-1.

Referring to structure 1 again, L and L_q are independently a chemical bond or the residue of a linking group. The phrase "residue of a linking group" as used herein refers to a moiety that remains, results, or is derived from the reaction of a protein reactive group with a reactive site on the protein. The phrase "protein reactive group" as used herein refers to any group which can react with functional groups typically found on proteins. However, it is specifically contemplated that such protein reactive groups can also react with functional groups typically found on nonprotein biomolecules. Thus the linking groups useful in the practice of this invention derive from those groups which can react with any biological molecule containing an immunoreactive group, whether or not the biological molecule is a protein, to form a linking group between the immunoreactive group and the oligonucleotide containing species as described below.

Preferred linking groups are derived from protein reactive groups selected from but not limited to:

(1) A group that will react directly with amine, alcohol, or sulfhydryl groups on the protein or biological molecule containing the immunoreactive group,

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for example, active halogen containing groups including, for example, chloromethylphenyl groups and chloroacetyl [ClCH₂C(=O)-] groups, activated 2-(leaving group substituted)-ethylsulfonyl and ethylcarbonyl groups such as 2-chloroethylsulfonyl and 2-chloroethylcarbonyl; vinylsulfonyl; vinylcarbonyl; epoxy; isocyanato; isothiocyanato; aldehyde; aziridine; succinimidoxycarbonyl; activated acyl groups such as carboxylic acid halides; mixed anhydrides and the like; and other groups known to be useful in conventional photographic gelatin hardening agents;

(2) A group that can react readily with modified proteins or biological molecules containing the immunoreactive group, i.e., proteins or biological molecules containing the immunoreactive group modified to contain reactive groups such as those mentioned in (1) above, for example, by oxidation of the protein to an aldehyde or a carboxylic acid, in which case the "linking group" can be derived from protein reactive groups selected from amino, alkylamino, arylamino, hydrazino, alkylhydrazino, arylhydrazino, carbazido, semicarbazido, thiocarbazido, thiosemicarbazido, sulfhydryl, sulfhydrylalkyl, sulfhydrylaryl, hydroxy, carboxy, carboxyalkyl and carboxyaryl. The alkyl portions of said linking groups can contain from 1 to about 20 carbon atoms. The aryl portions of said linking groups can contain from about 6 to about 20 carbon atoms;

(3) A group that can be linked to the protein or biological molecule containing the immunoreactive group, or to the modified protein as noted in (1) and (2) above by use of a crosslinking agent. The residues of certain useful crosslinking agents, such as, for example, homobifunctional and heterobifunctional gelatin hardeners, bisepoxides, and bisisocyanates can become a

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part of, i.e., a linking group in, the protein-
(oligonucleotide-containing species) conjugate during
the crosslinking reaction. Other useful crosslinking
agents, however, can facilitate the crosslinking, for
5 example, as consumable catalysts, and are not present in
the final conjugate. Examples of such crosslinking
agents are carbodiimide and carbamoylonyl crosslinking
agents as disclosed in U.S. Patent No. 4,421,847 and the
ethers of U.S. Patent No. 4,877,724. With these
10 crosslinking agents, one of the reactants, such as the
immunoreactive group, must have a carboxyl group and the
other, such as the oligonucleotide containing species,
must have a reactive amine, alcohol, or sulfhydryl
group. In amide bond formation, the crosslinking agent
15 first reacts selectively with the carboxyl group, then
is split out during reaction of the thus "activated"
carboxyl group with an amine to form an amide linkage
between the protein and oligonucleotide containing
species, thus covalently bonding the two moieties. An
20 advantage of this approach is that crosslinking of like
molecules, e.g., proteins with proteins or
oligonucleotide containing species with themselves is
avoided, whereas the reaction of, for example, homo-
bifunctional crosslinking agents is nonselective and
25 unwanted crosslinked molecules are obtained.

Preferred useful linking groups are derived from
various heterobifunctional cross-linking reagents such
as those listed in the Pierce Chemical Company
Immunotechnology Catalog - Protein Modification Section,
30 (1991 and 1992).

Useful non-limiting examples of such reagents
include:

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	Sulfo-SMCC	Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate.
5	Sulfo-SIAB	Sulfosuccinimidyl (4-iodoacetyl)aminobenzoate.
	Sulfo-SMPB	Sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate.
10	2-IT	2-Iminothiolane.
	SATA	N-Succinimidyl S-acetylthioacetate.

15

In addition to the foregoing description, the linking groups, in whole or in part, can also be comprised of and derived from nucleotides and residues of nucleotides, both naturally occurring and modified. Particularly useful, non-limiting reagents for incorporation of modified nucleotide moieties containing reactive functional groups, such as amine and sulfhydryl groups, into an oligonucleotide sequence of this invention are commercially available from, for example, Clonetech Laboratories Inc. (Palo Alto California) and include Uni-Link AminoModifier (Catalog # 5190), Biotin-ON phosphoramidite (Catalog # 5191), N-MNT-C6-AminoModifier (Catalog # 5202), AminoModifier II (Catalog # 5203), DMT-C6-3'Amine-ON (Catalog # 5222), C6-ThiolModifier (Catalog # 5211), and the like. In one aspect, linking groups of this invention are derived from the reaction of a reactive functional group such as an amine or sulfhydryl group as are available in the above Clonetech reagents, one or more of which has been incorporated into an oligonucleotide sequence of this invention, with, for example, one or more of the previously described protein reactive groups such as heterobifunctional protein reactive groups, one or more of which has been incorporated into an immunoreagent of this invention.

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Referring to Structure 1 again, I and I₁ independently comprise an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units wherein said contiguous sequence contains therein one or more members of a family of homologous contiguous sequences, the individual homologs of said family being comprised of from 12 to about 30 nucleotide units, the individual homologs of said family of sequences, both individually or as a set of homologous sequences so comprised being hereinafter sometimes referred to as "the Sequence", and contiguous sequences of six or more nucleotide units of said oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous nucleotide units anywhere in structure 1. Members of the set of homologous contiguous sequences which comprise "the Sequence" can be found in both the sequence I and the sequence I₁; at least one such sequence is common to both I and I₁.

The oligonucleotide sequence of I and I₁ can be comprised of DNA, of RNA, or of purine and pyrimidine base modified DNA or RNA, or of backbone modified DNA or RNA such as methyl phosphonate or thiophosphonate or carbohydrate modified DNA or RNA analogs, whole or partially modified, or of combinations thereof as long as a complementary oligonucleotide sequence incorporated into the radioactive targeting moiety described below can hybridize to said oligonucleotide sequence to form a hybrid which exhibits a T_m (melting temperature) greater than about 37°C. Preferred oligonucleotides are DNA and RNA. Especially preferred is DNA comprised of A, T, G, and C nucleotide units.

In a preferred embodiment, the oligonucleotide sequence I and I₁ can be comprised of double stranded DNA or RNA. That is, the oligonucleotide sequence may be comprised of complementary DNA or RNA which forms a

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double helix molecule. The complementary oligonucleotide sequence incorporated into the radioactive targeting moiety, composed of DNA or RNA, then hybridizes to one or the other of the strands of the double stranded DNA or RNA comprising I and I_i. In this way, the complementary oligonucleotide sequence incorporated into the radioactive targeting moiety interacts with the duplex DNA or RNA of I and I_i in such a way as to form triplex (triple helix) DNA, triplex RNA, or a triplex DNA-RNA hybrid.

Preferred non-limiting examples of oligonucleotide sequences of which the "Sequence" can be comprised are shown below. The following sequences comprise a set of homologous oligonucleotide sequences which when considered individually or in any combination comprise a set herein defined as the "Sequence":

- (i) TTATGGACGGAG (SEQ ID NO:1);
- (ii) TTATGGACGGAGA (SEQ ID NO:2);
- (iii) TTATGGACGGAGAA (SEQ ID NO:3);
- (iv) TTATGGACGGAGAAG (SEQ ID NO:4);
- (v) TTATGGACGGAGAAGC (SEQ ID NO:5);
- (vi) TTATGGACGGAGAAGCT (SEQ ID NO:6);
- (vii) TTATGGACGGAGAAGCTA (SEQ ID NO:7); and
- (viii) TTATGGACGGAGAAGCTAA (SEQ ID NO:8).

Of course, sequence (viii) contains sequence (vii) which contains sequence (vi) which contains sequence (v), and so on. Thus, in structure 1, if I contains, for example sequence (iii), and an I_i contains, for example, sequence (v), then both I and I_i contain at least sequence (iii). Another I_i in structure 1 can contain (viii), in which case it would also contain (i) through (vii) as well as (viii). In this case, all three sequences would contain at least (iii) [as well as (i) and (ii)], and the two I_i's would contain at least (v) [as well as (i) through (iv)]. In this regard, an

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oligonucleotide that is comprised of a contiguous sequence of nucleotides, which sequence being complementary to at least sequence (i), would hybridize to all sequences (i) through (viii) as would any member of a set of contiguous complementary sequences, the individual members of which are comprised of the sequence complementary to any of (i) through (viii). Such a set of contiguous complementary sequences can comprise cI as will be described below.

Another set of preferred homologous oligonucleotide sequences which can comprise the "Sequence" is:

- (ix) CGGAGAAGCTAA (SEQ ID NO:9);
- (x) ACGGAGAAGCTAA (SEQ ID NO:10);
- (xi) GACGGAGAAGCTAA (SEQ ID NO:11);
- (xii) GGACGGAGAAGCTAA (SEQ ID NO:12);
- (xiii) TGGACGGAGAAGCTAA (SEQ ID NO:13);
- (xiv) ATGGACGGAGAAGCTAA (SEQ ID NO:14);
- (xv) TATGGACGGAGAAGCTAA (SEQ ID NO:15); and
- (xvi) TTATGGACGGAGAAGCTAA (SEQ ID NO:8).

An especially preferred sequence is:
TTATGGACGGAGAAGCTAA (SEQ ID NO:8).

Two or more of the oligonucleotide sequences of this invention can be tandemly linked by means of chemical bonds, by linking groups such as described above, or by spacing groups as described below. The sequential order of nucleotides in the oligonucleotide sequences of this invention can be from the 5' to the 3' end or from the 3' to the 5' end. Attachment of the oligonucleotide sequences of this invention via linking groups as described above to the immune reactive group as described above can be accomplished via 3' or via 5' sites or via derivatives attached to 3' or 5' sites of the oligonucleotide.

As discussed elsewhere, the "Sequence" may also be composed of a double stranded DNA or RNA. That is, the

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"Sequence" may consist of complementary oligonucleotides which noncovalently interact to form double stranded DNA or RNA. Attachment of this double stranded nucleic acid to the immune reactive group as described above can be accomplished via 3' or via 5' sites or via derivatives attached to 3' or 5' sites of the oligonucleotide.

Referring to structure 1 again, Q_i is a spacing group which separates and links two or more oligonucleotide sequences of this invention. Q_i can be comprised of a linking group as defined above, alone, or in combination with a nucleotide or an oligonucleotide comprised of 2 to about 20 nucleotide units, the sequence of which is not self-associating or such that contiguous sequences of six or more nucleotide units comprised therein do not hybridize with any other contiguous sequences of six or more contiguous nucleotide units anywhere in structure 1. Q_i can also be comprised of residues of an amino acid group, a peptide group, or a poly(alkylene oxide) group such as a poly(ethylene glycol) group. It is contemplated that each spacing group can be linked to from two to about six oligonucleotide sequences at least two of which containing the Sequence of this invention. Preferably, the spacing group is linked to two oligonucleotide sequences each of which contain the Sequence of this invention. Preferably, the spacing group is an oligonucleotide sequence.

Non limiting examples of preferred spacing groups are oligonucleotides comprised of the following sequences:

ACT;
ACTC;
ACTCT;
CTC;
TCTC; and

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CTCTC.

An especially preferred spacing group is an oligonucleotide: ACTCTC.

Of course, the condition described above, i.e.,
5 that the oligonucleotide sequence of this invention does not comprise a self associating sequence, still applies when considering the selection of oligonucleotide spacing groups linked in combination with the oligonucleotide Sequence groups.

10 In structure 1, a is from 0 to about 6, preferably an integer from 1 to about 6, more preferably one to about 4, and most preferably one or two.

In structure 1, p is an integer from 1 to 10, preferably 1 to about 6, and more preferably 1 to 3. It
15 is also contemplated that mixtures of immunoreactive proteins comprised of mixtures of Z modified as defined in structure 1 together with Z not so modified will also be useful in this invention. In this case, the bulk mixture properties of "p" of such mixtures would
20 comprise fractional values from about zero to about 10. Preferably, the bulk p values would be from about 0.1 to about 10.0, more preferably from about 0.2 to about 5.0, and most preferably from about 0.4 to about 3.

In structure 1, E is an end capping group. E is
25 preferably a nucleotide group that is modified so as to reduce or prevent the action of exonuclease enzymatic activity on the oligonucleotide sequence. It can be a 3'- or 5'-phosphate linked ribose group containing one or more substituents such as an alkyl group of 1 to
30 about 10 carbon atoms, or an ether group such as alkyl or aryl or aralkyl or substituted aryl or aralkyl ether wherein the alkyl groups contain from 1 to about 10 carbon atoms and such alkyl or aryl groups may contain or be substituted by substituents containing oxygen,
35 nitrogen or sulfur atoms, or a poly(alkylene oxidyl)

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group, preferably at the 5'- or 3'- position, respectively, or elsewhere on the ribose group, which substituent will reduce or prevent the action of exonuclease enzymatic activity. A phosphate ester
5 comprised of such entities is also useful, as well as a phosphate ester or modified ribose comprised of elements of a suitable linking group as defined above. E can also be comprised of Z or it can be attached to Q by elements of L as defined above to form a cyclic
10 structure. E can also be comprised of compounds with a two carbon-one nitrogen atom internucleoside linkage. Preferably, E is comprised of a poly(alkylene glycol) phosphate diester. With respect to E, the poly(alkylene glycol) moiety can have from 2 to about 100 repeating
15 units. Preferably, the poly(alkylene glycol) is a poly(ethylene glycol). A currently preferred poly(alkylene glycol) phosphate diester is a tetra(ethylene glycol) phosphate diester, hereinafter sometimes referred to as a "Teg" or "Teg unit". Such
20 poly(alkylene glycol) phosphate diesters can be linked in tandem to each other to form a dimer phosphate ester sequence, a trimer sequence, a tetramer sequence, and so forth. One or two such units is preferred. Such units can also be attached to residues of Q, L, I, or Z
25 described above. A preferred end group E is comprised of a Teg unit linked by a phosphate ester bond to a nucleotide such as T.

In the context of this invention, the term "modified nucleotide moiety" is intended to mean a
30 chemical entity which is comprised of one or more chemical groups that are analogous to one or more portions of a naturally occurring nucleotide or of a residue of a naturally occurring nucleotide. A "modified nucleotide moiety" is comprised of that
35 chemical entity which exclusively remains when one or

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more chemical bonds, of which said naturally occurring nucleotide is otherwise comprised when considered as an independent chemical entity, is altered, modified, or replaced to comprise one or more covalent bonds to one
5 or more other chemical entities, or is comprised of that chemical entity which exclusively remains after removal or deletion of a portion, such as, for example, a purine or pyrimidine base portion, a hydroxyl portion, a ribose portion, and the like or combinations thereof, of the
10 naturally occurring nucleotide in one location simultaneously with said replacement of another portion of said nucleotide. Particularly useful, non-limiting examples of modified nucleotide moieties are comprised of reactive functional groups, such as amine and
15 sulfhydryl groups. They can be commercially available such as, for example, those modified nucleotide moieties and precursors thereto which are available from Clonetech Laboratories Inc. (Palo Alto, California). Said modified nucleotide moieties and precursors thereto
20 include Uni-Link AminoModifier (Catalog #5190), Biotin-ON phosphoramidite (Catalog #5191), N-MNT-C6-AminoModifier (Catalog #5202), AminoModifier II (Catalog #5203), DMT-C6-3'Amine-ON (Catalog #5222), C6-ThiolModifier (Catalog #5211), and the like. One or
25 more of said moieties can be incorporated into an oligonucleotide sequence comprising this invention. In one aspect, linking groups of this invention are derived from the reaction of a reactive functional group such as an amine or sulfhydryl group as are available in the
30 above Clonetech reagents, one or more of which has been incorporated into an oligonucleotide sequence of this invention, with, for example, one or more of the previously described protein reactive groups such as heterobifunctional protein reactive groups, one or more

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of which has been incorporated into an immunoreagent of this invention.

5 A "modified nucleotide moiety" can comprise a nucleotide moiety that is modified so as to reduce or prevent the action of exonuclease enzymatic activity on the oligonucleotide sequence. It can be a 3'- or 5'-phosphate linked ribose or a 3'- or 5'-phosphate linked 2'-deoxyribose group containing one or more substituents such as an alkyl group of 1 to about 10 carbon atoms, or 10 an ether group such as alkyl or aryl or aralkyl or substituted aryl or aralkyl ether wherein the alkyl groups contain from 1 to about 10 carbon atoms and such alkyl or aryl groups may contain or be substituted by substituents containing oxygen, nitrogen or sulfur 15 atoms, or a poly(alkylene oxidyl) group, preferably at the 5'- or 3'-ribose position, respectively, or elsewhere on the ribose group, which substituent will reduce or prevent the action of exonuclease enzymatic activity. A "modified nucleotide moiety" comprising a 20 phosphate ester comprised of said substituents is also useful, as well as a phosphate ester or modified ribose comprised of elements of a suitable linking group as defined above. A "modified nucleotide moiety" can also be comprised of Z or it can be attached to Q by elements 25 of L as defined above to form a cyclic structure. A "modified nucleotide moiety" can also be comprised of compounds with a two carbon-one nitrogen atom internucleoside linkage.

30 Preferably, a "modified nucleotide moiety" is comprised of a poly(alkylene glycol) phosphate diester. With respect to said "modified nucleotide moiety", the poly(alkylene glycol) moiety can have from 2 to about 100 repeating units. Preferably, the poly(alkylene glycol) is a poly(ethylene glycol). A currently 35 preferred poly(alkylene glycol) phosphate diester is a

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tetra(ethylene glycol) phosphate diester, hereinafter sometimes referred to as a "Teg" or "Teg unit". Such poly(alkylene glycol) phosphate diesters can be linked in tandem to each other to form a dimer phosphate ester sequence, a trimer sequence, a tetramer sequence, and so forth. One or two such units is preferred. Such units can also be attached to residues of Q, L, I or Z described herein. A preferred "modified nucleotide moiety" is comprised of a Teg unit linked by a phosphate ester bond to a nucleotide such as T.

Referring to the radioactive targeting immunoreagent described in Structure 2, preferred non-limiting examples of a set of oligonucleotides, cI, which are complementary to the members of the set of oligonucleotides comprising the "Sequence" of I in structure 1 include the DNA oligonucleotides:

(xvii) TTAGCTTCTCCG (SEQ ID NO:16);
(xviii) TTAGCTTCTCCGT (SEQ ID NO:17);
(xix) TTAGCTTCTCCGTC (SEQ ID NO:18);
(xx) TTAGCTTCTCCGTCC (SEQ ID NO:19);
(xxi) TTAGCTTCTCCGTCCA (SEQ ID NO:20);
(xxii) TTAGCTTCTCCGTCCAT (SEQ ID NO:21);
(xxiii) TTAGCTTCTCCGTCCATA (SEQ ID NO:22); and
(xxiv) TTAGCTTCTCCGTCCATAA (SEQ ID NO:23)

Another set of preferred homologous oligonucleotide sequences which can comprise a set complementary to the "Sequence" of structure 1 is:

(xxv) CTCCGTCCATAA (SEQ ID NO:24);
(xxvi) TCTCCGTCCATAA (SEQ ID NO:25);
(xxvii) TTCTCCGTCCATAA (SEQ ID NO:26);
(xxviii) CTTCTCCGTCCATAA (SEQ ID NO:27);
(xxix) GCTTCTCCGTCCATAA (SEQ ID NO:28);
(xxx) AGCTTCTCCGTCCATAA (SEQ ID NO:29);
(xxxi) TAGCTTCTCCGTCCATAA (SEQ ID NO:30);
(xxxii) TTAGCTTCTCCGTCCATAA (SEQ ID NO:23)

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An especially preferred complementary sequence is comprised of:

TTAGCTTCTCCGTCATAA (SEQ ID NO: 23)

Of course, as is well known in the art, the complementarity of the above cI oligonucleotide sequences of structure 2 with respect to the previously listed I oligonucleotide sequences of structure 1 depends on the relative orientation of each, i.e., whether the sequences of I and of cI are constructed from 5' to 3' or 3' to 5', or vice versa, respectively. Preferably, the T_m of a hybridized complex formed between the respective I and cI sequences is greater than 37°C.

In a preferred embodiment, the oligonucleotide sequence cI can be comprised of double stranded DNA or RNA. That is, the oligonucleotide sequence may be comprised of complementary DNA or RNA which forms a double helix molecule. The complementary oligonucleotide sequence incorporated into the non-radioactive targeting immunoreagent, composed of DNA or RNA, then hybridizes to one or the other of the strands of the double stranded DNA or RNA comprising cI. In this way, the complementary oligonucleotide sequence incorporated into the non-radioactive targeting immunoreagent interacts with the duplex DNA or RNA of cI in such a way as to form triplex (triple helix) DNA, triplex RNA, or a triplex DNA-RNA hybrid.

Q_{cI} in structure 2 is a spacing group; Q_{cI} can be selected from Q_I as described for structure 1. Preferably, Q_{cI} is comprised of an oligonucleotide comprised of a contiguous sequence of from 2 to about 30 nucleotides, the sequence of which is not self-associating and such that a contiguous sequence of six or more nucleotide units comprised therein does not hybridize with any other contiguous sequence of six or

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more contiguous nucleotide units anywhere in structure 2. Q_{CT} can also be comprised of an oligonucleotide, preferably a sequence such as (xxvii) to (xxxii) above which is complementary to the "Sequence" in structure 1. Preferably, Q_{CT} is comprised of one or two such sequences.

L_1 , L_2 , and L_3 in structure 2 are independently a chemical bond, preferably a phosphate ester bond, or a linking group which are defined as L and L_0 in the above structure 1. L_1 , L_2 , and L_3 can also be independently comprised of components of Q_{CT} .

W_1 , W_2 , and W_3 in structure 2 are residues of chelating groups. The chelating groups of this invention can comprise the residue of one or more of a wide variety of chelating agents that can have a radionuclide associated therewith. As is well known, a chelating agent is a compound containing donor atoms that can combine by coordinate bonding with a metal atom to form a cyclic structure called a chelation complex or chelate. This class of compounds is described in the Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 5, 339-368.

The residues of suitable chelating agents can be independently selected from polyphosphates, such as sodium tripolyphosphate and hexametaphosphoric acid; aminocarboxylic acids, such as ethylenediaminetetraacetic acid, N-(2-hydroxyethyl)ethylene-diaminetriacetic acid, nitrilotriacetic acid, N,N-di(2-hydroxyethyl)glycine, ethylenebis(hydroxyphenylglycine) and diethylenetriamine pentacetic acid; 1,3-diketones, such as acetylacetone, trifluoroacetylacetone, and thenoyltrifluoroacetone; hydroxycarboxylic acids, such as tartaric acid, citric acid, gluconic acid, and 5-sulfosalicylic acid; polyamines, such as ethylenediamine, diethylenetriamine,

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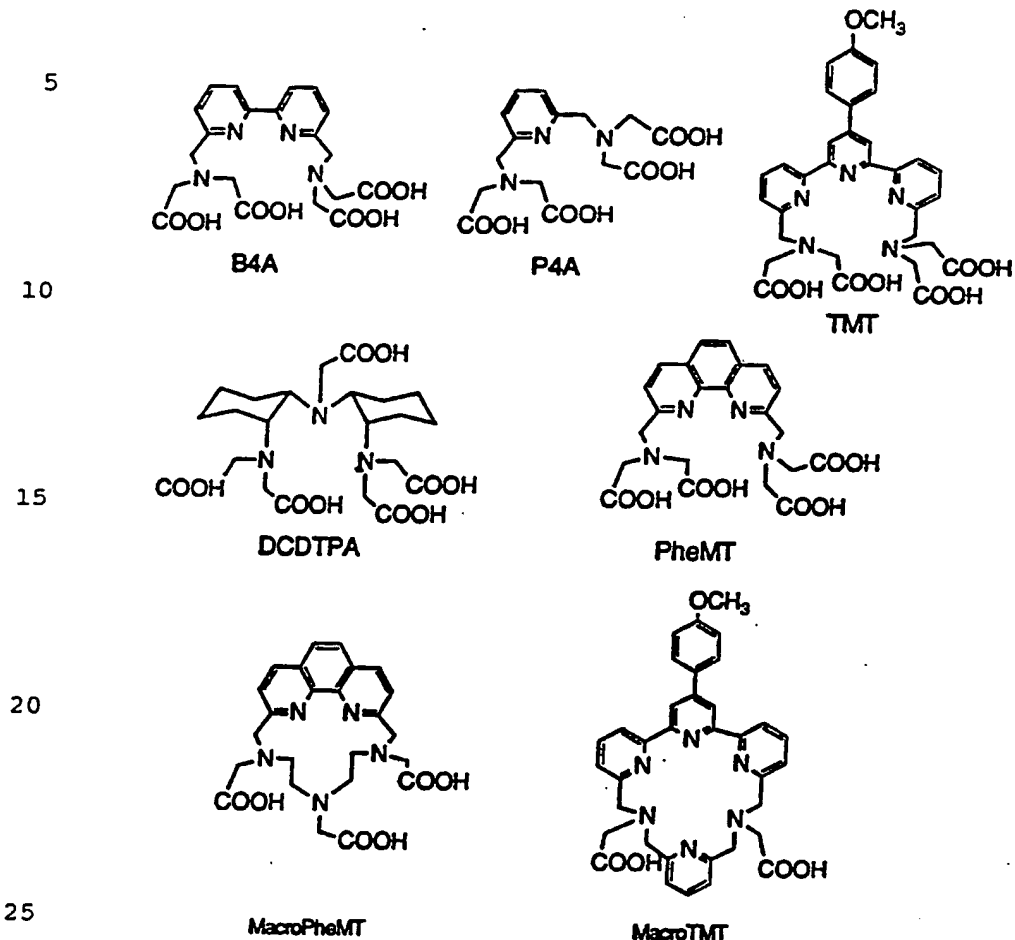
triethylenetetramine, and triaminotriethylamine;
aminoalcohols, such as triethanolamine and N-(2-
hydroxyethyl)ethylenediamine; aromatic heterocyclic
bases, such as 2,2'-dipyridyl, 2,2'-diimidazole,
5 dipicoline amine and 1,10-phenanthroline; phenols, such
as salicylaldehyde, disulfopyrocatechol, and
chromotropic acid; aminophenols, such as 8-
hydroxyquinoline and oximesulfonic acid; oximes, such as
dimethylglyoxime and salicylaldoxime; peptides
10 containing proximal chelating functionality such as
polycysteine, polyhistidine, polyaspartic acid,
polyglutamic acid, or combinations of such amino acids;
Schiff bases, such as disalicylaldehyde 1,2-
propylenediimine; tetrapyrroles, such as
15 tetraphenylporphyrin and phthalocyanine; sulfur
compounds, such as toluenedithiol, meso-2,3-
dimercaptosuccinic acid, dimercaptopropanol,
thioglycolic acid, potassium ethyl xanthate, sodium
diethyldithiocarbamate, dithizone, diethyl
20 dithiophosphoric acid, and thiourea; synthetic
macrocylic compounds, such as dibenzo[18]crown-6,
(CH₂)₆-[14]-4,11-diene-N₄, and (2.2.2-cryptate); and
phosphonic acids, such as nitrilotrimethylene-phosphonic
acid, ethylenediaminetetra(methylenephosphonic acid),
25 and hydroxyethylidenediphosphonic acid, or combinations
of two or more of the above agents.

Preferred residues of chelating agents contain
polycarboxylic acid groups and include: ethylenediamine-
N, N, N', N'-tetraacetic acid (EDTA); N, N, N', N'', N'''-
30 diethylene-triaminepentaacetic acid (DTPA); 1,4,7,10-
tetraazacyclododecane-N, N', N'', N'''-tetraacetic acid
(DOTA); 1,4,7,10-tetraazacyclododecane-N, N', N''-triacetic
acid (DO3A); 1-oxa-4,7,10-triazacyclododecane-N, N', N''-
triacetic acid (OTTA); trans(1,2)-
35 cyclohexanodiethylenetriamine pentaacetic acid (CDTPA);

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Preferred residues of chelating agents contain polycarboxylic acid groups and include the following:



In one aspect, other suitable residues of chelating agents are comprised of proteins modified for the chelation of metals such as technetium and rhenium as described in U.S. Patent No. 5,078,985, the disclosure of which is hereby incorporated by reference.

In another aspect, suitable residues of chelating agents are derived from N_3S and N_2S_2 containing compounds, as for example, those disclosed in U.S. Patent Nos. 4,444,690; 4,670,545; 4,673,562; 4,897,255;

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4,965,392; 4,980,147; 4,988,496; 5,021,556 and
5,075,099.

5 Other suitable residues of chelating agents are
described in PCT/US91/08253, the disclosure of which is
hereby incorporated by reference. In structure 2 above,
if W_1 , W_2 , and W_3 are comprised of the residue of
multiple chelating agents, such agents can be linked
together by a linking group such as described above in
structure 1.

10 The residue of the chelating agents W_1 , W_2 , and W_3
in structure 2 are independently linked to the
complementary oligonucleotide moiety cI or spacing group
 Q_{ci} through a chemical bond or a linking group, i.e., L_1 ,
 L_2 and L_3 in structure 2, above. Preferred linking
15 groups include nitrogen atoms in groups such as amino,
imido, nitrilo and imino groups; alkylene, preferably
containing from 1 to 18 carbon atoms such as methylene,
ethylene, propylene, butylene and hexylene, such
alkylene optionally being interrupted by 1 or more
20 heteroatoms such as oxygen, nitrogen and sulfur or
heteroatom-containing groups;
carbonyl;
sulfonyl;
sulfinyl;
25 ether;
thioether;
ester, i.e., carbonyloxy and oxycarbonyl;
thioester, i.e., carbonylthio, thiocarbonyl,
thiocarbonyloxy, and oxythiocarboxy;
30 amide, i.e., iminocarbonyl and carbonylimino;
thioamide, i.e., iminothiocarbonyl and
thiocarbonylimino;
thio;
dithio;
35 phosphate;

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phosphonate;

urelene;

thiourelene;

urethane, i.e., iminocarbonyloxy, and oxycarbonylimino;

5 thiourethane, i.e., iminothiocarbonyloxy and

oxythiocarbonylimino;

an amino acid linkage, i.e., a

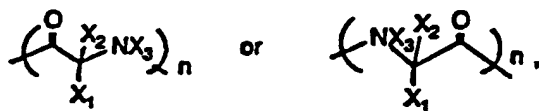
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group wherein $n=1$ and X_1 , X_2 , X_3 independently are H, alkyl, containing from 1 to 18, preferably 1 to 6 carbon atoms, such as methyl, ethyl and propyl, such alkyl optionally being interrupted by 1 or more heteroatoms such as oxygen, nitrogen and sulfur, substituted or unsubstituted aryl, containing from 6 to 18, preferably 6 to 10 carbon atoms such as phenyl, hydroxyiodophenyl, hydroxyphenyl, fluorophenyl and naphthyl, aralkyl, preferably containing from 7 to 12 carbon atoms, such as benzyl, heterocyclyl, preferably containing from 5 to 7 nuclear carbon and one or more heteroatoms such as S, N, P or O, examples of preferred heterocyclyl groups being pyridyl, quinolyl, imidazolyl and thienyl; heterocyclylalkyl, the heterocyclyl and alkyl portions of which preferably are described above; or a peptide linkage, i.e., a

30



35

group wherein $n>1$ and each X independently is represented by a group as described for X_1 , X_2 , X_3 above. Two or more linking groups can be used, such as, for

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example, alkyleneimino and iminoalkylene. It is contemplated that other linking groups may be suitable for use herein, such as linking groups commonly used in protein heterobifunctional and homobifunctional conjugation and crosslinking chemistry as described for L or L₀ in structure 1.

Especially preferred linking groups include unsubstituted or substituted phosphate ester groups containing amino groups which when linked to the residue of a chelating agent via an isothiocyanate group on the chelating agent form a thiourea group.

The linking groups can contain various substituents which do not interfere with the coupling reaction between chelate W₁, W₂, or W₃ and oligonucleotide cI or the spacing group. The linking groups can also contain substituents which can otherwise interfere with such reaction, but which during the coupling reaction, are prevented from so doing with suitable protecting groups commonly known in the art and which substituents are regenerated after the coupling reaction by suitable deprotection. The linking groups can also contain substituents that are introduced after the coupling reaction. For example, the linking group can be substituted with substituents such as halogen, such as F, Cl, Br or I; an ester group; an amide group; alkyl, preferably containing from 1 to about 18, more preferably, 1 to 4 carbon atoms such as methyl, ethyl, propyl, i-propyl, butyl, and the like; substituted or unsubstituted aryl, preferably containing from 6 to about 20, more preferably 6 to 10 carbon atoms such as phenyl, naphthyl, hydroxyphenyl, iodophenyl, hydroxyiodophenyl, fluorophenyl and methoxyphenyl; substituted or unsubstituted aralkyl, preferably containing from 7 to about 12 carbon atoms, such as benzyl and phenylethyl; alkoxy, the alkyl portion of

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which preferably contains from 1 to about 18 carbon atoms as described for alkyl above; alkoxyaralkyl, such as ethoxybenzyl; substituted or unsubstituted heterocyclyl, preferably containing from 5 to 7 nuclear carbon and heteroatoms such as S, N, P or O, examples of preferred heterocyclyl groups being pyridyl, quinolyl, imidazolyl and thienyl; a carboxyl group; a carboxyalkyl group, the alkyl portion of which preferably contains from 1 to 8 carbon atoms; or the residue of a chelating group.

In structure 2, M_1 , M_2 and M_3 are comprised of elements with oxidation states equal to or greater than +1, at least one of which is a radionuclide. Preferably M_1 , M_2 and M_3 are comprised of metal isotopes, preferably radioactive metal isotopes, sometimes herein referred to as metal radioisotopes, which radioisotopes are useful in therapeutic or in diagnostic imaging applications. Preferred metal radioisotopes are selected from, for example, Sc, Fe, Pb, Ga, Y, Bi, Mn, Cu, Cr, Zn, Ge, Mo, Tc, Ru, In, Sn, Re, Sr, Sm, Lu, Eu, Ru, Dy, Sb, W, Re, Po, Ta and Tl. Useful emissions from such radioisotopes comprise spontaneous emissions comprised of alpha emissions, beta emissions, gamma emissions, X-ray emission, positron emissions, and comprise such emissions as are induced by the processes of electron capture and internal conversion. Said emissions can be purely of one kind such as pure alpha, pure beta, pure gamma and the like, or of combinations of nuclear emissions such as beta and gamma emissions and the like.

Radioisotopes with emissions comprised, for example, of alpha radiation or of beta radiation are useful in therapeutic applications, especially in therapy of cancer. Useful isotopes in therapeutic applications include, for example, alpha radiation

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emitting isotopes such as, for example, ^{207}Pb , ^{211}Pb , ^{208}Pb , ^{212}Pb , ^{212}Bi , ^{207}Ti , and ^{223}Ra ; beta radiation emitting isotopes such as, for example ^{47}Sc , ^{66}Ga , ^{67}Cu , ^{77}As , ^{90}Y , ^{103}Rh , ^{109}Pd , ^{111}Ag , ^{121}Sn , ^{127}Te , ^{143}Pr , ^{149}Pm , ^{153}Sm , ^{161}Tb , ^{166}Ho , ^{169}Er , ^{177}Lu , ^{188}Re , ^{186}Re , ^{191}Os and ^{199}Au ; and isotopes which emit radiation as a result of the processes of electron capture and internal conversion such as, for example, ^{97}Ru , $^{177\text{m}}\text{Sn}$, ^{199}Sb , ^{128}Ba and ^{197}Hg . Radioisotopes especially preferred in therapeutic applications include ^{212}Pb , ^{212}Bi , ^{90}Y , ^{177}Lu , ^{186}Re , and ^{188}Re . Most preferred is ^{90}Y .

Radioisotopes with emissions comprised of, for example, gamma radiation or of positron radiation are useful in diagnostic imaging applications, especially in diagnostic imaging of cancer. Useful isotopes in diagnostic imaging applications include, for example, gamma radiation emitting isotopes such as ^{47}Sc , ^{51}Cr , ^{67}Cu , ^{67}Ga , ^{97}Ru , $^{99\text{m}}\text{Tc}$, ^{111}In , $^{117\text{m}}\text{Sn}$, ^{141}Ce , ^{167}Tm , ^{199}Au , ^{87}Y and ^{203}Pb ; and positron radiation emitting isotopes such as ^{44}Sc , ^{48}V , ^{64}Cu , ^{66}Ga , ^{68}Ge , ^{72}As , ^{86}Y and ^{89}Zr . Radioisotopes especially preferred in diagnostic imaging applications include ^{64}Cu , $^{99\text{m}}\text{Tc}$, ^{111}In and ^{87}Y . Most preferred are $^{99\text{m}}\text{Tc}$ and ^{111}In .

In another aspect, other suitable radionuclides can be incorporated, for example, by covalent bonding, into QcI and include radioactive isotopes of halogens such as radioactive isotopes of iodine, for example, ^{123}I , ^{124}I , ^{125}I and ^{131}I as well as radioactive isotopes of astatine such as ^{211}At .

Methods of generating an image useful in the diagnostic imaging of, for example, cancer in a mammal comprise detecting emissions imagewise from radioisotopes as employed in the compositions and methods of this invention. Said image generating methods comprise the use of, for example, a collimated

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camera detector such as a gamma camera commonly employed in radioimmunosciintigraphy (RIS), and the use of linked X-ray detectors commonly employed in positron emission tomography (PET) and in single photon emission tomography (SPET).

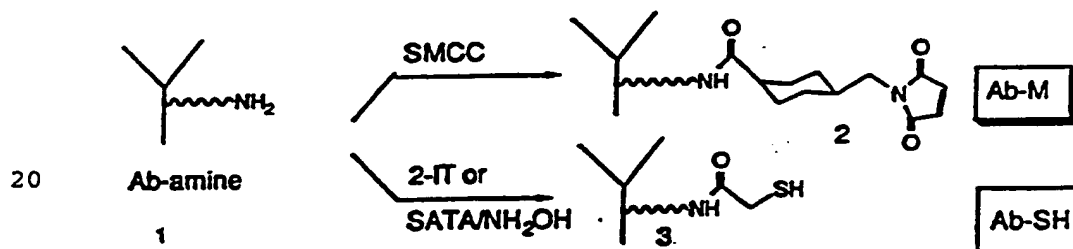
In structure 2, x, y, and z are independently zero or 1 provided that at least one of x, y, or z is one; and

w and b are zero or an integer from 1 to about 4.

Preferred compositions can be prepared as outlined in the schemes that follow.

Scheme 1

Derivatization of antibody amine groups with heterobifunctional linking reagents SMCC, 2-IT, or SATA.



In scheme 1, the protein (antibody, enzyme, receptor) is chemically modified for covalent coupling to a thiolated oligonucleotide or to a maleimido group containing oligonucleotide. Chemical modification is effected using a bifunctional cross linking agent, preferably a heterobifunctional cross linking agent having both a group capable of reacting with protein functional groups (e.g. amine) and also having a further group capable of reacting with thiol groups. The latter is selected from haloacetyl, halo-acetamidyl, maleimido, and activated disulfide functions.

Maleimido and thioalkyl groups are introduced to antibody by utilizing the heterobifunctional linkers,

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sulfosuccinimido-4-(M-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC), 2-iminothiolane (2 IT), or succinimidyl-S-acetylthioacetate(SATA). The reaction of antibody with linking agent is for a time sufficient to introduce about 0.5-3 linking agent molecules per antibody molecule. The derivatized antibody is purified using a gel filtration column, and more preferably Sephadex G-25.

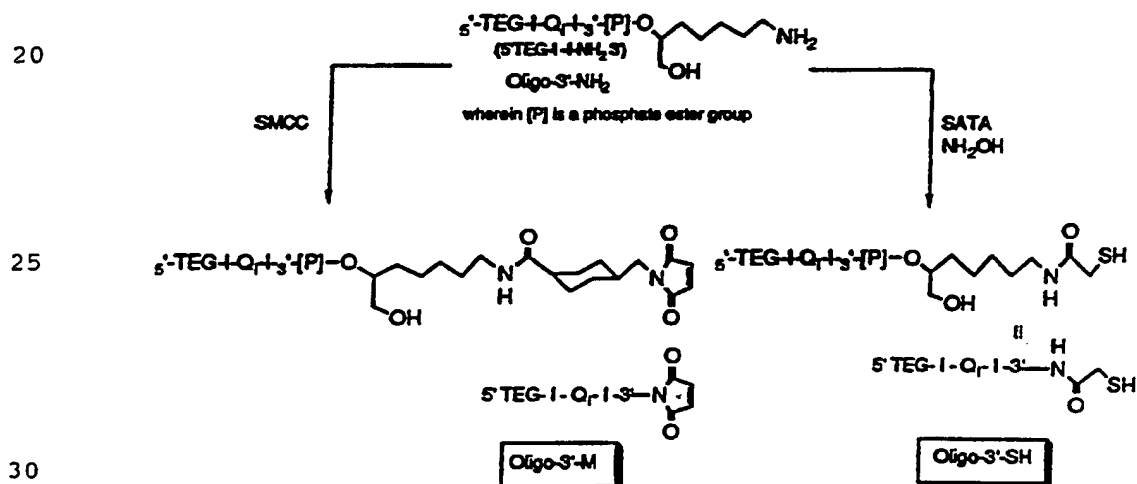
Non-limiting examples of preferred protein conjugates prepared are listed below:

ING-1-NH-CO-cyclohexane-CH₂-Maleimide

$$\text{ING-1-NH-C(=NH)} - (\text{CH}_2)_3\text{-SH}$$
$$\text{ING-1-NH-CO-CH}_2\text{-SH}$$

Scheme 2

Derivatization of oligonucleotide "I-Q_r-I" via amine groups with heterobifunctional linking reagents SATA and SMCC.



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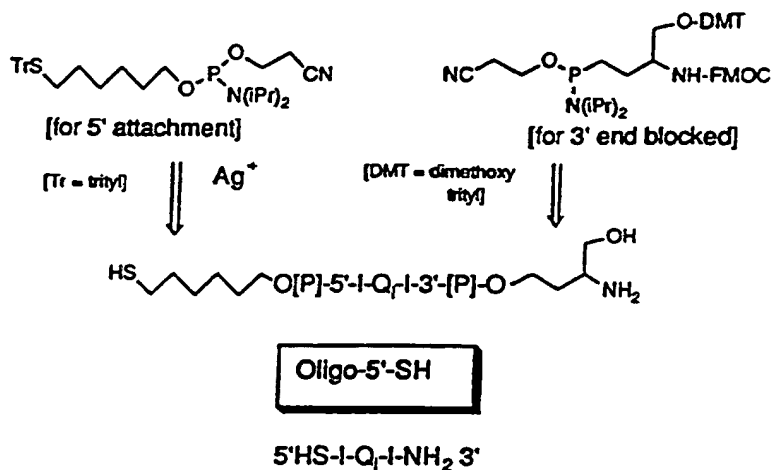
Scheme 3

Derivatization of oligonucleotide "I-Q_I-I" with amine groups and with sulfhydryl groups for use with heterobifunctional linking reagents.

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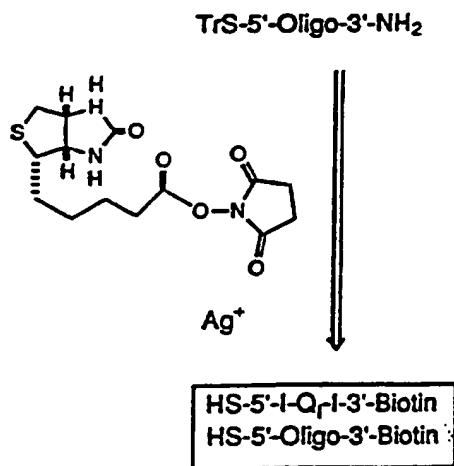
Scheme 4

Derivatization of oligonucleotide "5'-TrS-I-Q_I-I-3'-NH₂" with biotin for use with heterobifunctional linking reagents.

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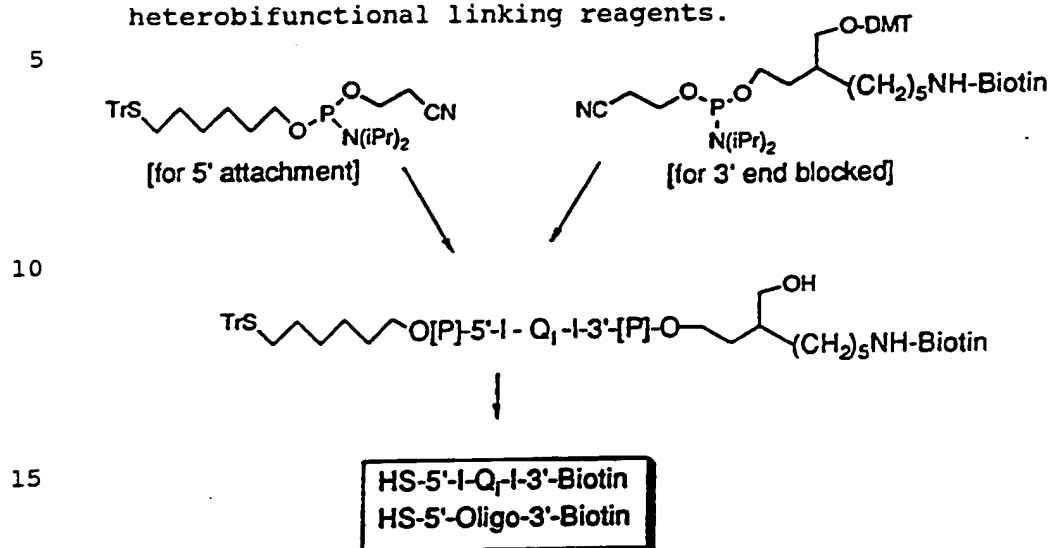


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Scheme 5

Derivatization to produce "5'-HS-I-Q₁-I-3'-biotin" with biotinylated phosphoramidite reagent for use with heterobifunctional linking reagents.



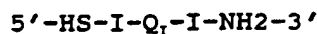
Oligonucleotides and modified oligonucleotides are synthesized according to standard methods known in the art. Derivatizations of oligonucleotides I-Q₁-I were achieved utilizing the reaction of 5'-TEG-oligomer-NH₂-3' with SATA or SMCC affording oligomer-3'-SH or oligomer-3'-M (see Scheme 2).

The following preferred oligonucleotides were used:
5'-Teg-TCTTATGGACGGAGAAGCTAAACTCTCTTATGGACGGAGAAGCTAAATCT-3'-amine-T, (SEQ ID No: 31; where the underlined portion denotes the spacer);

and

TTATGGACGGAGAAGCTAA (SEQ ID NO: 8)

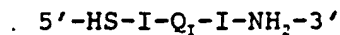
These oligomers are also derivatized to afford a bifunctionalized oligomer:



via introduction of bifunctional reagents at the 5'- and 3'-positions, as shown in Scheme 3.

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The introduction of biotin at the 3'-position is also achieved by the reaction of

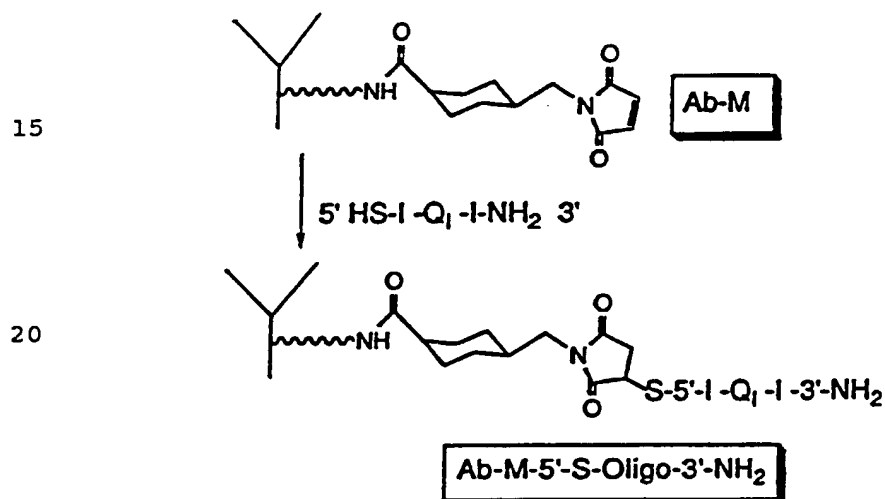


with biotin-imidocarboxylate as shown in Scheme 4.

5 There are useful spacers with a protected amine, thiol, or carboxy on one end and a phosphoramidite at the other (see Scheme 5).

The applications of these spacers are shown in reactions below.

10 Scheme 6

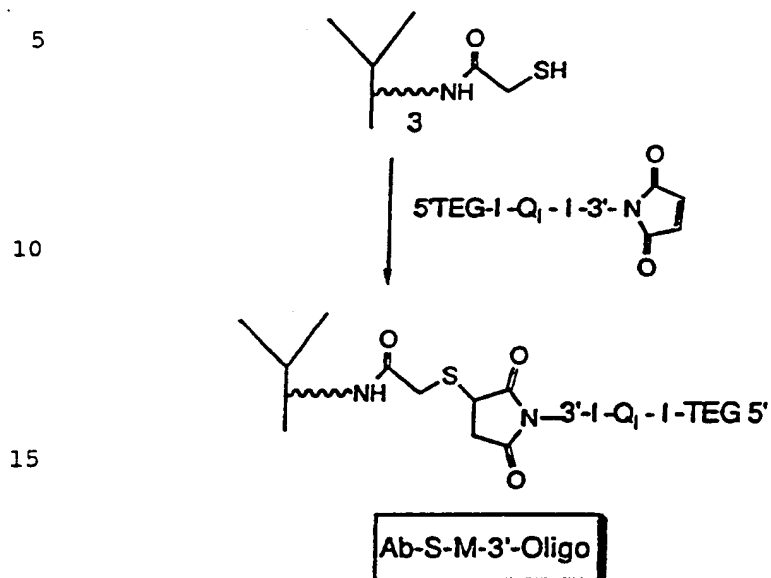


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Scheme 7

Assembly of Ab-SH + Oligo-3'-M



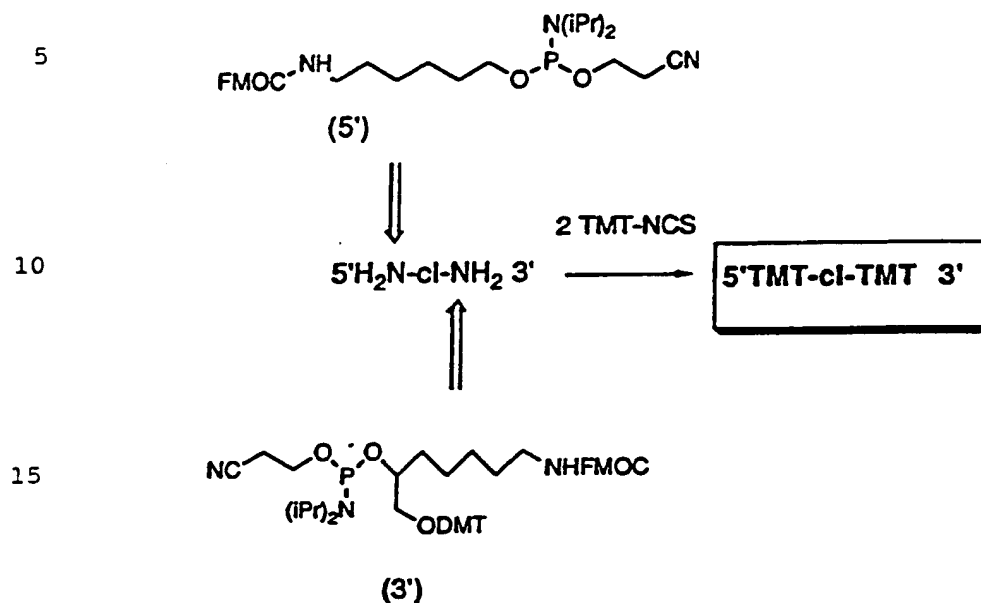
The modified maleimido antibody (2; Ab-M, Scheme 1) and the thiolated oligonucleotide (5'-HS-I-Q₁-I-NH₂-3') can be assembled to yield the modified antibody-oligonucleotide conjugate (Ab-M-5'-S-succinimido-I-Q₁-I-NH₂-3') as shown in Scheme 6. Similarly, AB-S-M-3'-I-Q₁-I-TEG-5' is prepared from AB-SH and 5'-TEG-I-Q₁-I-maleimide-3' as shown in Scheme 7.

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Scheme 8

Complementary Oligonucleotide "5'-TMT-cl-TMT-3' "



wherein Ab = Antibody, protein;

Oligo = oligonucleotide;

cl = complementary oligonucleotide sequence;

25 I-Q₁-I = oligonucleotide; and

TMT = terpyridine chelates.

The reaction of 5'-H₂N-cl-NH₂-3' with the excess (2 mol) TMT-NCS affords the desired 5'-TMT-cl-TMT-3' as shown in Scheme 8.

30 There are other useful linking agents with a protected amine, thiol, or carboxy on one end and a phosphoramidite at the other. The applications of these agents are shown in the Examples.

35 In a preferred embodiment, an effective dose of a radioactive targeting reagent as described above in a

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pharmaceutically acceptable medium is prepared by exposing a composition of a complementary oligonucleotide sequence containing one or more chelating groups as described above to a composition containing a radioactive metal isotope such that the molar amount of said radionuclide metal isotope is less than the molar amount of said chelating group, said duration of exposure lasting an effective time to permit uptake of said metal isotope into said chelating agent.

In a preferred embodiment, an effective dose of a non-radioactive targeting immunoreagent as described above in a pharmaceutically acceptable medium is administered to a patient and said non-radioactive targeting immunoreagent is allowed to accumulate at the target site such as at a tumor site in said patient. Subsequently, an effective dose of a radioactive targeting reagent as described above in a pharmaceutically acceptable medium is administered to said patient, and said radioactive targeting reagent is allowed to accumulate at the target site, said target site being the non-radioactive targeting immunoreagent accumulated at said tumor site in said patient.

The present invention includes one or more of the immunoreagents of this invention formulated into compositions together with one or more non-toxic physiologically acceptable carriers, adjuvants or vehicles which are collectively referred to herein as carriers, for parenteral injection for oral administration in solid or liquid form, for rectal or topical administration, or the like.

The compositions can be administered to humans and animals either orally, rectally, parenterally (intravenous, intramuscularly or subcutaneously), intracisternally, intravaginally, intraperitoneally,

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intravesically, locally (powders, ointments or drops), or as a buccal or nasal spray.

5 Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols
10 (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the
15 case of dispersions and by the use of surfactants.

These compositions may also contain adjuvants such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the action of microorganisms can
20 be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by
25 the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such
30 solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol and silicic acid, (b)
35 binders, as for example, carboxymethylcellulose,

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alignates, gelatin, polyvinylpyrrolidone, sucrose and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates and sodium carbonate, (e) solution retarders, as for example paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate or mixtures thereof. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethyleneglycols, and the like.

Solid dosage forms such as tablets, dragees, capsules, pills and granules can be prepared with coatings and shells, such as enteric coatings and others well known in the art. They may contain opacifying agents, and can also be of such composition that they release the active compound or compounds in a certain part of the intestinal tract in a delayed manner. Examples of embedding compositions which can be used are polymeric substances and waxes.

The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the

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active compounds, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butyleneglycol, dimethylformamide, oils, in particular, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethyleneglycols and fatty acid esters of sorbitan or mixtures of these substances, and the like.

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents. Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like.

Compositions for rectal administrations are preferably suppositories which can be prepared by mixing the compounds of the present invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethyleneglycol or a suppository wax, which are solid at ordinary temperatures but liquid at body temperature and therefore, melt in the rectum or vaginal cavity and release the active component.

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays and inhalants. The active component is admixed under sterile conditions with a physiologically acceptable carrier and any preservatives, buffers or propellants as may be required. Ophthalmic

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formulations, eye ointments, powders and solutions are also contemplated as being within the scope of this invention.

5 Actual dosage levels of active ingredient in the compositions of the present invention may be varied so as to obtain an amount of active ingredient that is effective to obtain a desired therapeutic response for a particular composition and method of administration. The selected dosage level therefore depends upon the
10 desired therapeutic effect, on the route of administration, on the desired duration of treatment and other factors.

The total daily dose of the compounds of this invention administered to a host in single or divided
15 dose may be in amounts, for example, of from about 100 picomol to about 5 micromols per kilogram of body weight. Dosage unit compositions may contain such amounts of such submultiples thereof as may be used to make up the daily dose. It will be understood, however,
20 that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of administration, rates of absorption and excretion, combination with other drugs and the severity of the
25 particular disease being treated.

In another embodiment, the present invention is directed to a method of diagnosis comprising the administration of a diagnostic imaging effective amount of the compositions of the present invention to a mammal
30 in need of such diagnosis. A method for diagnostic imaging for use in medical procedures in accordance with this invention comprises administering to the body of a test subject in need of a diagnostic image an effective diagnostic image producing amount of the above-described
35 compositions.

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In this method, an effective diagnostic image producing amount of a non-radioactive targeting immunoreagent as described above in a pharmaceutically acceptable medium is administered to a patient and said non-radioactive targeting immunoreagent is allowed to accumulate at the target site such as at a tumor site in said patient. Subsequently, a diagnostic imaging effective dose of a radioactive targeting reagent as described above in a pharmaceutically acceptable medium is administered to said patient, and said radioactive targeting reagent is allowed to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said tumor site in said patient. The image pattern can then be visualized.

In addition to human patients, the test subjects can include mammalian species such as rabbits, dogs, cats, monkeys, sheep, pigs, horses, bovine animals and the like.

After administration of the compositions of the present invention, the subject mammal is maintained for a time period sufficient for the administered compositions to be distributed throughout the subject and enter the tissues of the mammal. A sufficient time period is generally from about 1 hour to about 2 weeks or more and, preferably from about 2 hours to about 1 week.

The following examples further illustrate the invention and are not to be construed as limiting of the specification and claims in any way. Specific embodiments of the invention are illustrated in the following examples.

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EXAMPLES

Example 1

Oligonucleotide Design

5 A targeting immunoreagent of this invention as described in Structure 1 comprised of an antibody Z, linking groups L and L₀, and an oligonucleotide comprised of a sequence I, a spacing group Q_r, a second sequence I_i, and an end capping group E was designed as follows.

10 A sample oligonucleotide with the following sequence (SEQ ID NO: 32) was analyzed for conformity to the criteria described previously for Structure 1 wherein groups L, I, Q_r, I_i, and L₀ and E are as represented below:

15 L I Q_r I_i L₀ E
 5'TC|TTATGGACGGATCCGCTAA|TC|TTATGGACGGATCCGCTAA|TCT3'
 (SEQ ID NO 32)

20 Analysis of this oligonucleotide sequence using "Oligo" computer software (National Biosciences) revealed it to contain 5 regions of self-complementarity. All such regions were hairpin loops with differing degrees of overlap. The sum of the negative free energy changes (ΔG's) for these self-complementary regions was -28.3 kcal/mole which predicted a melting temperature of 86°C in 1.0 molar salt for this oligonucleotide. The most stable region of self-complementarity (ΔG = -15 kcal/mol) contained 8 base pairs most of which correspond to a BAM-1 restriction site palindrome. In the presence of these regions of self-complementarity an oligonucleotide sequence complementary to this sequence cannot hybridize successfully to its full length. Modifications to this nucleotide sequence were then made in accordance with

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the criteria outlined for structure 1 to replace selected bases in regions of self-complementarity such that excessive base pairing was removed in iterative analysis. Additional nucleotides were also inserted
 5 into the spacer sequence to ensure that, in helical conformations, the terminal groups of two complementary sequences, cI, when hybridized to the two sequences I and I₁ were orthogonal to each other.

In this manner, a new oligonucleotide (SEQ ID NO:
 10 33) with the following sequence was designed, and then synthesized and conjugated to an immunoreactive molecule (i.e., to an antibody):

	L		I		Q ₁		I ₁		L ₀	E
15	5X-TC	TTATGGACGGAGAAGCTAA	ACTCTC	TTATGGACGGAGAAGCTAA	TCTYT-3'					
	(SEQ ID NO: 33)									

This oligonucleotide (SEQ ID NO: 33) is comprised of two copies of the same nucleotide sequence (i.e., I = I₁ = TTATGGACGGAGAAGCTAA; SEQ ID NO: 8) linked through 6
 20 nucleotide spacer (Q₁ = ACTCTC). The groups L and L₀E at the 5' or the 3' end are comprised of groups X and Y which can be comprised of either an amine-containing group (such as, for example, from those available from Clontech Industries, which amine-containing groups being
 25 sometimes hereinafter cryptically referred to as "NH₂" or as "amine") or one or more TEG groups as described above.

Thus, in one embodiment, this oligonucleotide, cryptically referred to as 5'-Teg-I-Q₁-I-3'-amine, is:
 30 5'-Teg-TCTTATGGACGGAGAAGCTAACTCTCTTATGGACGGAGAAGCTAATCT-3'-NH₂-T
 (SEQ ID NO: 31)
 5'-Teg-I-Q₁-I-3'amine.

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In another embodiment, this oligonucleotide, cryptically referred to as 5'-amine-I-Q_I-I-3'-Teg, is:

5'-H₂N-TCTTATGGACGGAGAAGCTAAACTCTCTTATGGACGGAGAAGCTAATCT-3'-Teg-T

(SEQ ID NO: 34)

5

5'-amine-I-Q_I-I-3'-Teg.

Example 2

(2a) Synthesis of I-Q_I-I; 5'-Teg-I-Q_I-I-3'-NH₂

10

(SEQ ID NO: 31)

The oligonucleotide was prepared on an Applied Biosystems oligonucleotide synthesizer by the trityl-on protocol as directed by the equipment manufacturer using 2-deoxynucleotide phosphoramidite reagent precursors (5'-dimethoxytrityl cytidine-3'-O-phosphoramidite, 5'-dimethoxytrityl adenosine-3'-O-phosphoramidite, 5'-dimethoxytrityl guanosine-3'-O-phosphoramidite, and 5'-dimethoxytrityl thymidine-3'-O-phosphoramidite from Applied Biosystems). Clonetech's Uni-link Amino Modifier was used as the precursor to the 3'-amine group. The TEG group, a (tetra(ethylene glycol)) phosphate diester linked by a phosphate ester bond to 5'-dimethoxytrityl thymidine-3'-O-phosphoramidite as disclosed in WO/92/02534 was used at the 5'-end of the strand. Following synthesis of the whole oligonucleotide, the base protecting groups and solid support were removed with ammonium hydroxide and the resulting 5' protecting group was removed with 3% trichloroacetic acid. The oligonucleotide was desalted and further purified by elution down an OPEC Cartridge (Clonetech) with deionized water. Electrophoresis on a 12% polyacrylamide gel was used to further purify the oligonucleotides into upper and lower bands. The DNA band was visualized by U.V. shadowing. It was cut out, minced, and extracted

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with a buffer comprised of 10mM Tris HCl and 1mM EDTA at pH 7.5 at 4°C for 24 hours. The gel pieces were then removed by centrifugation, and the DNA was purified through a spun column of Sephadex G-25. The
5 concentration of oligonucleotide was estimated using absorbance at 260 nm.

(2b) Synthesis of 5'-H₂N-I-Q₁-I-3'-Teg (SEQ ID NO: 34)

10 This oligonucleotide was prepared on an Applied Biosystems oligonucleotide synthesizer by the trityl-off protocol as directed by the equipment manufacturer using 2-deoxynucleotide phosphoramidite reagent precursors (5'-dimethoxytrityl cytidine-3'-O-phosphoramidite, 5'-
15 dimethoxytrityl adenosine-3'-O-phosphoramidite, 5'-dimethoxytrityl guanosine-3'-O-phosphoramidite, and 5'-dimethoxytrityl thymidine-3'-O-phosphoramidite from Applied Biosystems). Clontech's 6 carbon monomethoxytrityl AminoModifier (N-MMT-C6-AminoModifier)
20 was used as the precursor of the 5'-amine group. The TEG group was used as the terminal group at the 3' end. The base protecting group and solid support were removed with ammonium hydroxide and the oligonucleotide was further purified by polyacrylamide gel electrophoresis.

25.

(2c) Synthesis of 5'-Trityl-S-I-Q₁-3'-NH₂

The oligonucleotide sequence of Example 2a minus the Teg group was prepared on an Applied Biosystems
30 oligonucleotide synthesizer by the trityl-on protocol as directed by the equipment manufacturer using 2-deoxynucleotide phosphoramidite reagent precursors (5'-dimethoxytrityl cytidine-3'-O-phosphoramidite, 5'-dimethoxytrityl adenosine-3'-O-phosphoramidite, 5'-
35 dimethoxytrityl guanosine-3'-O-phosphoramidite, and 5'-

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dimethoxytrityl thymidine-3'-O-phosphoramidite from Applied Biosystems). Clontech's Uni-link Amino Modifier was used as the precursor to the 3'-amine group. Clontech's C6-ThioModifier was used as the precursor to the 5'-thiol group (which in herein referred to cryptically as 5-S) added to the sequence in place of T_{eg} in Example 2a. Following synthesis of the whole oligonucleotide, the base protecting groups and solid support were removed with ammonium hydroxide. The oligonucleotide was desalted and further purified by elution down an OPEC Cartridge (Clontech) with deionized water. The concentration of oligonucleotide was estimated using absorbance at 260 nm.

(2d) Preparation of fluorescent 5'-T_{eg}-I-Q_i-I-3'-NH-Cy5.18

A sample (30 nmoles) of 5'-T_{eg}-I-Q_i-I-3'-NH₂, prepared according to Example 2a, was evaporated to dryness and redissolved in 500 μ L 0.1 M bicarbonate buffer at pH9 by vortexing. This sample was then added to a vial containing the dried succinimidyl ester of the dye Cy5.18 (Biological Detection Systems; Pittsburg PA). After thorough mixing the reaction was allowed to proceed at room temperature for one hour with frequent mixing. The product, 5'-T_{eg}-I-Q_i-I-3'-NH-Cy5.18, was purified by elution from a Sephadex G-25 column with distilled water.

Example 3

(3a) Synthesis of cI diamine, 5'-NH₂-cI-3'-NH₂

An oligonucleotide, cI, containing the nucleotide sequence 5'-X-TTAGCTTCTCCGTCATAAYT-3' (SEQ ID

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NO: 35) complementary to I was prepared on an Applied Biosystems oligonucleotide synthesizer as outlined in Example 2a. The 3' (Y) and 5' (X) amine-containing groups were incorporated as directed by the equipment manufacturer using Uni-link Amino Modifier (Clontech) for the precursor to the 3'-amine group, and Clontech's 6 carbon monomethoxytrityl AminoModifier (N-MMT-C6-AminoModifier: Catalog # 5202) as precursor to the 5'-amine group. After final deblocking and removal from the solid support, the protecting groups were removed with ammonium hydroxide, the amine-functionalized oligonucleotide was purified by elution down an OPEC Cartridge (Clontech) with deionized water. The oligonucleotide was further purified by polyacrylamide gel electrophoresis or reverse-phase HPLC. The concentration of oligonucleotide was estimated using absorbance at 260 nm.

(3b) Synthesis of I diamine, 5'-NH₂-I-3'-NH₂

An oligonucleotide, I, containing the nucleotide sequence 5'X-TTATGGACGGAGAAGCTAAYT-3' (SEQ ID NO: 36) was prepared on an Applied Biosystems oligonucleotide synthesizer as outlined in Example 3a. The 3' (Y) and 5' (X) amine-containing groups were incorporated as directed by the equipment manufacturer using Uni-link Amino Modifier (Clontech) for the precursor to the 3'-amine group, and Clontech's 6 carbon monomethoxytrityl AminoModifier (N-MMT-C6-AminoModifier: Catalog # 5202) as precursor to the 5'-amine group. After final deblocking and cleavage from the solid support, the protecting groups were removed with ammonium hydroxide, the amine-functionalized oligonucleotide was purified by elution down an OPEC Cartridge (Clontech) with deionized water. The oligonucleotide was further

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purified by polyacrylamide gel electrophoresis or reverse-phase HPLC. The concentration of oligonucleotide was estimated using absorbance at 260 nm.

5 (3c) Synthesis of cI

 An oligonucleotide, cI, containing the nucleotide sequence 5'-TTAGCTTCTCCGTCCATAA-3' (SEQ ID NO: 23) complementary to (I) was prepared on an Applied Biosystems oligonucleotide synthesizer as outlined in
10 Example 2a. After final deblocking and cleavage from the solid support, the protecting groups were removed with ammonium hydroxide, and the oligonucleotide was purified by elution down an OPEC Cartridge (Clonetech) with
15 deionized water. The oligonucleotide was further purified by polyacrylamide gel electrophoresis. The concentration of oligonucleotide was estimated using absorbance at 260 nm.

20 (3d) Synthesis of I

 An oligonucleotide, I, containing the nucleotide sequence 5'-TTATGGACGGAGAAGCTAA-3' (SEQ ID NO: 8) was prepared on an Applied Biosystems oligonucleotide
25 synthesizer as outlined in Example 3c. After final deblocking and cleavage from the solid support, the protecting groups were removed with ammonium hydroxide, and the oligonucleotide was purified by elution down an OPEC Cartridge (Clonetech) with deionized water. The
30 oligonucleotide was further purified by polyacrylamide gel electrophoresis. The concentration of oligonucleotide was estimated using absorbance at 260 nm.

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(3e) Preparation of fluorescent cI and I

5 A sample (30 nmoles) of cI diamine, prepared according to Example 3a, was evaporated to dryness and redissolved in 500 μ L 0.1 M bicarbonate buffer at pH9 by vortexing. This sample was then added to a vial containing the dried succinimidyl ester of the dye Cy5.18 (Biological Detection Systems; Pittsburg PA). After thorough vortexing the reaction was allowed to proceed at room temperature for one hour with frequent mixing. 10 CY5.18 labeled cI was purified by elution from a Sepahadex G-25 column with distilled water. A sample of Cy5.18 labeled I diamine (Example 3b) was prepared in the same manner.

15

Example 4

Preparation of 32 P radiolabeled oligonucleotides

20

Solutions (50 pmoles DNA in deionized water) of oligonucleotide (cI) from Example 3c or oligonucleotide (I) from Example 3d, were diluted into a buffer containing 0.5M Tris (pH 7.6), 0.1M $MgCl_2$, 50 mM dithiothreitol, 1mM spermine and 1mM EDTA at room temperature. A sample (20 units) of T4 polynucleotide kinase was added to each sample which was then incubated at 37°C. Radiolabeling was initiated by the addition of 50 pmoles of [γ - 32 P]ATP (3 Ci/ μ mole) to give a final total volume of 50 μ L of each sample. After 60 minutes the reaction was halted by the addition of 2 μ L of 0.5M EDTA. The radiolabeled DNA was extracted once with phenol/chloroform, precipitated with ice-cold ethanol and redissolved in 50 mMTris/EDTA buffer (pH 7.9).

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Finally the ^{32}P -cI or ^{32}P -I were passed down a Sephadex G-50 column to remove any unincorporated [γ - ^{32}P]ATP.

Example 5

5

Hybridization of ^{32}P radiolabeled cI or I to I-Q₁-I

10 A 5 pmole sample ^{32}P -labeled cI solution (from Example 4) was mixed with increasing amounts (0.375 to 12 pmoles) of I-Q₁-I (from Example 2a) in PBS at 37°C for one hour. A 5 μL aliquot from each of these hybridizations was removed, mixed with SDS buffer, and run on a 12% PAGE gel. Autoradiographs of the gels revealed that each I-Q₁-I was capable of binding to 2
15 molecules of cI. Identical experiments using non-complementary, ^{32}P -labeled I (Example 4) failed to show hybridization to I-Q₁-I.

Example 6

20

(6a) Annealing of cI to I-Q₁-I

A sample of I-Q₁-I (2 μmoles), prepared according to Example 2a, was mixed with increasing amounts of cI
25 (100:5 to 100:200, I-Q₁-I:cI), prepared by the method of Example 3c, in 6SSC buffer at room temperature. The mixtures were loaded into a sample cuvette and analysed by UV light (260 nm) in a Cary 13 instrument while the cuvette temperature was ramped up from 30°C to 90°C and
30 then back down to 30°C. Analysis of the data revealed the presence of binary complexes (cI:I-Q₁-I and/or I-Q₁-I:cI) at concentrations of cI below equimolar with respect to I-Q₁-I and with a melting transition temperature (T_m) of around 48°C. At higher

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concentrations of cI, a ternary complex (cI:I-Q₁-I:cI) with a T_m of 72.5°C could be seen.

(6b) Hybridization of TMT-cI-TMT to I-Q₁-I

5

Similar experiments using cI-TMT (see Example 8a below) demonstrated the annealing of TMT-cI-TMT to I-Q₁-I with a ternary complex T_m of 74°C.

10

Example 7

Preparation of ³⁵S-labeled TMT-NCS

15 A suspension of about 40 μmoles of TMT amine (PCT US91/08253) in 650 μL methanol is stirred at room temperature and deionized water is added dropwise (about 70 μL) until a clear pale yellow solution develops. The solution is cooled in an ice bath to 10°C and about 60 μmoles ³⁵S-thiophosgene is added dropwise over about 3
20 minutes. A precipitate of TMT isothiocyanate forms and the solution is stirred continuously for a further 2.5 hours. The solution and precipitate are concentrated to near dryness on a rotovap under reduced pressure (~ 15mm Hg) at room temperature. The near-dry solid is
25 diluted further with about 500 - 750 μL methanol and is stirred until the solids appear homogeneous. The solids of TMT isothiocyanate are then collected by filtration and rinsed repeatedly with methanol. The product is dried overnight in a vacuum chamber.

30

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Example 8

(8a) Preparation 5'-H₂N-cI-3'-NH₂ conjugated to a chelating agent, TMT, to form TMT-cI-TMT.

5

To 300 nmoles oligonucleotide 5'-H₂N-cI-3'-NH₂ solution of Example 3a in 500 ul 1.0M carbonate/bicarbonate buffer at pH 9.0 was added 12 mg of TMT isothiocyanate (PCT US91/08253). The reaction mixture was vortex mixed and kept at 37°C for 2 hours and at room temperature for overnight. The resulting reaction mixture was quenched with ethanolamine (15 uM) and the product was purified by Sephadex G-25 column chromatography, eluting with deionized water.

10

The number of TMTs per molecule of cI diamine was quantified by an assay using the time resolved fluorescence of chelated Europium metal.

15

(8b) General procedure for labelling of Inq-1-TMT and TMT-cI-TMT conjugates with fluorescent metals.

20

Binding of lanthanides such as europium (3+) to chelating agents that contain an aromatic moiety held close to the co-ordination sphere can lead to "sensitized" fluorescence wherein light is absorbed through the aromatic system and the energy is transferred to the metal. The metal then produces emissions characterized by a very large Stokes shift and fluorescence lifetimes of up to several seconds. The fluorescence at 615 nm is measured at a time-delay of 400 microseconds after an excitation pulse at 340 nm. This time delay is critical for high sensitivity measurements since short lived background fluorescence is efficiently eliminated, and up to a 1,500 fold

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enhancement in sensitivity over normal Eu-fluorescence is achievable.

In this method, a known amount of Ing-1-TMT or TMT-cI-TMT conjugate is titrated with increasing amounts of added EuCl₃ in an aqueous buffer. Thus, one microliter of a solution containing 1-30 picomoles (0.15 µg - 5µg) of the conjugate is added, in duplicate, to wells in a Costar EIA/RIA 96-well plate containing a precalculated amount of Tris.HCl buffer (pH 7.4). The buffer volume is derived by subtracting from 99 the volume in microliters of aqueous EuCl₃ (typically 10⁻⁴ M to 10⁻⁶ M in Tris.HCl buffer). The total volume in each well is thereby fixed at 100 microliters. Aqueous EuCl₃ is then added to the buffered solution of the conjugate. The plate is then covered and shaken at low speed for one hour. The time resolved fluorescence is then measured using a Delfia 1232 time-resolved fluorimeter (Wallac Inc.) and the data are analyzed. It is found that each conjugated TMT molecule chelates one Europium ion and that the TMT-cI-TMT conjugate binds two Europium ions per molecule of conjugate.

(8c) Preparation of 5'-NH₂-cI-3'-NH₂ Conjugated to ³⁵S-labeled TMT-NCS

³⁵S-labeled TMT-NCS (prepared as in Example 7) is substituted for the 12 mg of TMT isothiocyanate (PCT US91/08253) in the method of Example 8a and the reaction carried out as described above. The number of TMTs per molecule of cI is quantified by counting the TMT-cI-TMT product in a liquid scintillation counter optimized to detect ³⁵S. From a knowledge of the concentration of cI and the specific activity of ³⁵S-thiophosgene, the number of TMT molecules per cI diamine may be calculated.

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Example 9

(9a) Preparation of a Yttrium (^{90}Y) radiolabeled-
oligonucleotide, ^{90}Y -TMT-cI-TMT- ^{90}Y .

5

A solution of the oligonucleotide TMT conjugate (TMT-cI-TMT) from Example 8a in deionized water at room temperature was treated with a solution of $^{90}\text{YCl}_3$ (>500 Ci/mg: Amersham) in 0.5 M sodium acetate buffer at pH 6.0 to a specific activity of 0.1 Ci/pmol for one hour at room temperature. The labeling efficiency was determined by removing 1.0 μL of the sample and spotting it on to a Gelman ITLC-SG strip. The strip was developed in a glass beaker containing 0.1 M sodium citrate, pH 6.0 for a few minutes until the solvent front reached three-quarters of the way to the top of the paper. The strip was then inserted into a System 200 Imaging Scanner (Bioscan) which had been optimized for ^{90}Y and was controlled by a Compaq 386/20e computer. In this system unbound ^{90}Y migrates at the solvent front. The TMT-cI-TMT chelated in excess of 98 % of the added radioactivity.

25 (9b) Administration of ^{90}Y -TMT-cI-TMT- ^{90}Y to Nude
Mice

TMT-cI-TMT, labeled with ^{90}Y to a specific activity of 28 $\mu\text{Ci}/28 \mu\text{g}$, was injected into a 25g nude mouse bearing a subcutaneous tumor in its right flank. At time intervals after injection, blood samples were taken from the tail and counted for ^{90}Y radioactivity in a liquid scintillation counter. The results revealed that more than 99% of the injected dose of ^{90}Y was removed from the blood stream in the first 30 minutes following

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injection. After 2 hours following injection the radioactivity in the blood leveled off and a minute fraction (< 0.01% of injected dose) continued to circulate during the next 22 hours. These data confirm that, in the absence of a hybridizing site recognizing the cI sequence, radiolabeled cI-TMT is lost quickly from the circulation.

Example 10

10

(10a) Preparation of Antibody-Maleimide with Sulfo-SMCC (ING-1-Maleimide)

A Sulfo-SMCC solution (108 nmoles) in PBS was added to a sample of a chimeric antibody (ING-1; 18 nmoles) solution in phosphate buffer (pH7). The resulting mixture was allowed to stand for 30 minutes with occasional mixing at room temperature. The reaction was stopped with 60 nmoles basic tris buffer. The reaction mixture was diluted with phosphate buffered saline, added to a prewashed PD-10 column, and eluted with PBS to afford ING-1-maleimide. This material was stored on ice until use.

20 (10b) Preparation of mercaptoalkyl-Antibody (ING-1-SH)

A sample of a chimeric antibody (ING-1; 5 nmoles) solution in 0.1 M carbonate buffer (pH 8.8) was mixed with 200 nmoles of an aqueous solution of 2-iminothiolane. The resulting mixture was allowed to stand for 30 min. with occasional mixing at room temperature. The reaction mixtures were diluted with phosphate buffed saline, added to a prewashed PD-10 column (Pharmacia), and eluted with PBS to afford

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mercaptoalkyl-ING-1. This material was stored on ice until use.

Example 11

5

(11a) Preparation of a mercaptoalkyl-I-Q₁-I using 2-iminothiolane, 5'-Teg-I-Q₁-I-3'-SH

10 A sample of a 5'-Teg-I-Q₁-I-3'-NH₂ (30 nmoles) solution in water was mixed with 1 M carbonate buffer (pH 9) to give a final buffer concentration of 890 mM. Into the buffered DNA was added 12 μ moles of an aqueous solution of 2-iminothiolane and these reactants were vortex mixed and kept at 37°C for 30 minutes. The
15 reaction mixture was quenched by the addition of 12 μ moles of ethanolamine, diluted with phosphate buffered saline, added to a prewashed NAP-25 column (Pharmacia), and eluted with PBS to afford 5'-Teg-I-Q₁-I-3'-NHC(NH₂⁺)CH₂CH₂CH₂SH. For use in conjugation to
20 maleimide-derivatized antibody, the product was eluted off the column directly into the antibody solution. Otherwise, mercaptoalkyl-I-Q₁-I was stored on ice until use.

25 (11b) Preparation of a mercaptoalkyl-I-Q₁-I using 2-iminothiolane, 5'-HS-I-Q₁-I-3'-Teg

A sample of 5'-H₂N-I-Q₁-I-3'-Teg (30 nmoles) was treated as in Example 11a to afford 5'-
30 HSCH₂CH₂CH₂(NH₂⁺)CHN-I-Q₁-I-Teg-3'.

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(11c) Preparation of an I-Q₁-I-Maleimide using
Sulfo-SMCC; 5'-Teg-I-Q₁-I-3'-Maleimide

5 An aqueous solution containing 20 nmoles of 5'-Teg-I-Q₁-I-3'-NH₂ (prepared as in Example 2a) was diluted into phosphate buffed saline. Sulfo-SMCC (100 nmoles) in PBS was added and the resulting mixture was allowed to stand for 30 min. with occasional mixing at room temperature. The reaction mixture was diluted with
10 phosphate buffed saline, added to a prewashed PD-10 column, and eluted with PBS to afford 5'-Teg-I-Q₁-I-3'-Maleimide. This material was stored on ice until use.

15 (11d) Preparation of an I-Q₁-I-Maleimide, 5'-Maleimide-I-Q₁-I-3'-Teg

5'-H₂N-I-Q₁-I-3'-Teg is reacted with sulfo-SMCC in the same way as in 11c to afford 5'-Maleimide-I-Q₁-I-3'Teg.

20

(11e) Preparation of 3'-mercapto-I-Q₁-I using SATA

25 An aqueous solution containing 50 nmoles of 5'-Teg-I-Q₁-I-3'-NH₂ (prepared as in Example 2a) was diluted into PBS and 500 nmoles of SATA (in DMSO) was added. After mixing and standing at room temperature for 60 min, the reaction mixture was diluted with PBS, and eluted from a NAP-10 column with PBS to afford 5'-Teg-I-Q₁-I-3'NH-CO-CH₂-S-CO-CH₃. The acetylthioacetylated
30 oligonucleotide was deprotected by the addition of 30 μ L of a pH 7.5 solution containing 100 mM sodium phosphate, 25 mM EDTA, 500 mM NH₄OH. The reaction proceeded for two hours at room temperature after which the material was again passed down a NAP-5 column by elution with PBS.

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The final product (5'-Teg-I-Q₁-I-3'NH-CO-CH₂-SH) was used immediately.

(11f) Preparation of 5'mercapto-I-Q₁-I using SATA

H₂N-5'-I-Q₁-I-3'-Teg is reacted with SATA in the same way as in Example 11e to afford 5'-HS-CH₂-OC-N-I-Q₁-I-3'Teg.

Example 12

(12a) Conjugation of 5'Teg-I-Q₁-I-3'NHC(NH₂⁺)CH₂CH₂CH₂SH to Antibody-Maleimide; ING-1-Maleimide-3'-S-I-Q₁-I-5'-Teg

A sample (108 nmoles) of 5'Teg-I-Q₁-I-3'-NHC(NH₂⁺)CH₂CH₂CH₂SH (prepared according to Example 11a) was eluted off a NAP-25 column directly into a solution of maleimide-derivatized ING-1 (18 nmoles) prepared according to Example 10a. After mixing, the reaction was allowed to proceed for 20 hours at 4°C. The reactants were then loaded into Centricon-100® concentration devices (Amicon), and centrifuged at 1000g for 25 minutes. The sample was resuspended in fresh PBS, and concentration by centrifugation was repeated a further 3 times until the ratio of optical densities at 260 nm and 280 nm was constant. The final product is ING-1-Maleimide-S-(CH₂)₃-C(NH₂⁺)NH-3'I-Q₁-I-5'Teg.

(12b) Conjugation of 5'-HSCH₂CH₂(NH₂⁺)CHN-I-Q₁-I-Teg-3' to Antibody-Maleimide, ING-1-Maleimide-5'-S-I-Q₁-I-Teg-3'

A sample (108 nmoles) of 5'-HSCH₂CH₂CH₂(NH₂⁺)CHN-I-Q₁-I-Teg-3' (prepared according to Example 11b) was

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conjugated to 18 nmoles of maleimide-derivatized ING-1 as in Example 12a to give ING-1-Maleimide-5'S-(CH₂)₃-C(NH₂⁺)NH-I-Q_I-I-3'Teg.

5 (12c) Assays on the ING-1-Maleimide-S-I-Q_I-I conjugates

The optical density of both ING-1-Maleimide-S-(CH₂)₃-C(=NH)NH-I-Q_I-I samples was examined in a spectrophotometer at 260 nm and 280 nm. The ratio of optical densities at these two wavelengths was calculated and, by using known extinction coefficients for the antibody and for the oligonucleotide (approximate molecular weight 16500) at each of these wavelengths, the number of oligonucleotide molecules was estimated to be between 1 and 2 I-Q_I-I per antibody.

The concentrations of ING-1 in the conjugate solutions were determined by the BioRad protein assay using bovine immunoglobulin as the protein standard. These data agreed well with the antibody concentrations determined by examination of the optical density of the conjugate at 280 nm once it had been corrected for absorbance due to the conjugated I-Q_I-I. Both these sets of data were further confirmed by subjecting the antibody-I-Q_I-I conjugates to acid digestion and amino acid analysis.

Antibody-I-Q_I-I conjugates were examined for their ability to bind to antigens on the surface of a human tumor cell line to which the antibody had been raised. The immunoreactivity of the conjugates was compared by flow cytometry with a standard preparation of the antibody before being subjected to modification and conjugation to I-Q_I-I. Target HT29 cells (a human adenocarcinoma cell line: ATTC) were grown to confluency in tissue culture flasks using McCoy's media

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supplemented with 10% fetal calf serum. The cells were harvested by scraping the flask walls with a cell scraper. Cells from many separate flasks were pooled, centrifuged to a pellet, resuspended at 5×10^5 /mL in a solution of ice-cold 50mM sodium phosphate with 150 mM sodium chloride buffer pH 7.4 (PBS) supplemented with 0.1% bovine serum albumin (Sigma) and 0.02% sodium azide (Flow buffer). The cells were washed in this same buffer and then counted. An antibody standard curve was constructed by diluting a stock solution of ING-1 with an irrelevant (non binding), isotype-matched control antibody (human IgG₁) to give a number of samples ranging in ING-1 content from 10% to 100%. The standard curve was made in flow buffer so that each sample contained 1.0 μ g antibody protein per mL. Samples from the standard curve and unknowns were then incubated with 5×10^5 HT29 cells at 4°C for 1 hour. After extensive washing to remove unbound antibody, the cells were resuspended in 100 μ L flow buffer and incubated at 4°C for 1 hour with goat-anti-human antibody labeled with fluoresceine isothiocyanate (FITC). After further washing in flow buffer the samples were analyzed by flow cytometry on a Coulter EPICS 753 flow cytometer. Fluorescence from FITC and propidium iodide (PI) was excited using the 488 nm emission line of an argon laser. The output was set at 500 mw in light regulation mode. Single cells were identified by 90 degree and forward angle light scatter. Analysis windows were applied to these parameters to separate single cells from aggregates and cell debris. Fluorescence from FITC and propidium were separated with a 550nm long pass dichroic filter and collected through a 530 nm band pass filter (for FITC), and a 635 nm band pass filter (for PI). Light scatter parameters were collected as integrated pulses and fluorescence was collected as log

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integrated pulses. Dead cells were excluded from the assay by placing an analysis window on cells negative for PI uptake. The mean fluorescence per sample (weighted average from 2500 cells) was calculated for each histogram. FITC calibration beads were analysed in each experiment to establish a fluorescence standard curve. The average fluorescence intensity for each sample was then expressed as the average FITC equivalents per cell. Immunoreactivity was calculated by comparing the average fluorescence intensity of the unknown sample with values from the standard curve. From the immunoreactivity assay, ING-1-Maleimide-S-(CH₂)₃-C(NH₂⁺)NH-3'I-Q₁-I 5'Teg was 67.8% as immunoreactive as the ING-1 standard and ING-1-Maleimide-S-(CH₂)₃-C(NH₂⁺)NH-5'I-Q₁-I 3'Teg was 81.5% as immunoreactive. In a separate set of experiments, immunoreactivity of ING-1-I-Q₁-I (3' conjugate) and ING-1-I-Q₁-I (5' conjugate) were determined to be 68% and 59% respectively.

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Table 1

	Preparation	Band	Apparent Mol Wt. (daltons)
5	ING-1-Maleimide-S-(CH ₂) ₃ -C(NH ₂ ⁺) NH-3'I-Q ₁ -I 5'Teg	1	240,000
		2	219,000
		3	200,000
		4	174,000
10	ING-1-Maleimide-S-(CH ₂) ₃ -C(NH ₂ ⁺) NH-5'I-Q ₁ -I 3'Teg	1	240,000
		2	224,000
		3	200,000
15		4	174,000
	ING-1 antibody pre-conjugate	1	174,000
20	Samples of these conjugates were also subjected to electrophoresis on Novex 6% polyacrylamide gels using SDS buffers in order to estimate their apparent molecular weight and the degree of heterogeneity of the preparation. Using standards of known molecular weight run on the same gel, a standard curve was constructed of the distance travelled (Rf) versus the log of the molecular weight. From this standard curve the relative molecular weights of the bands associated with each conjugate preparation were determined (see Table 1). The SDS PAGE gels of ING1-I-Q ₁ -I and ING-1 antibody demonstrated that the molecular weight of the ING-1-I-Q ₁ -I conjugates were higher than that of the antibody alone.		
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(12d) Conjugation of Teg-5'-I-Q₁-I-3'-Maleimide to mercaptoalkyl-Antibody

20 nmoles of Teg-5'-I-Q₁-I-3' Maleimide (from
5 Example 11c) were reacted with 5nmoles of mercaptoalkyl-
ING-1 (from Example 10b) in PBS pH 7. After mixing the
reaction was allowed to continue at 4°C for 16 hours to
afford ING-1-NH-CO-CH₂-S-Maleimide-3'-I-Q₁-I-5'Teg.
The reaction mixture was loaded into a Centricon-100®
10 concentration device and centrifuged at 1000g for 25
minutes. The sample was resuspended in fresh PBS and
concentration by centrifugation was repeated a further 3
times until the ratio of optical densities at 260 nm and
280 nm was constant.

15 Maleimide-5'-I-Q₁-I-3'Teg is reacted with
mercaptoalkyl-ING-1 in the same way to afford ING-1-NH-
CO-CH₂-S-Maleimide-5'-I-Q₁-I-3'Teg.

20 (12e) Conjugation of Teg-5'-I-Q₁-I-3'N-CO-CH₂SH to Antibody-Maleimide

A 6 nmole sample of ING-1-Maleimide (from Example
10a) in PBS was reacted with 40 nmoles of Teg-5'-I-Q₁-I-
3'NH-CO-CH₂SH (from Example 11e) at 4°C for 16 hours.
25 The reactants were diluted with PBS and eluted in PBS
from a PD-10 column to afford ING-1-Maleimide-S-CH₂-OC-
NH-3'-I-Q₁-I-5'Teg. The product was concentrated in a
Centricon-300' device by centrifugation at 1000g for 25
minutes. The sample was resuspended in fresh PBS and
30 concentration by centrifugation was repeated a further 3
times until the ratio of optical densities at 260 nm and
280 nm was constant.

35 HS-CH₂-OC-NH-5'-I-Q₁-I-3'Teg is reacted with ING-1-
Maleimide in the same way to afford ING-1-Maleimide-S-
CH₂-OC-NH-5'-I-Q₁-I-3'Teg.

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The optical density of these samples is examined in a spectrophotometer at 260 nm and 280 nm. The ratio of optical densities at these two wavelengths is calculated and, by using known extinction coefficients for the antibody and for the oligonucleotide (approx molecular weight 16500) at each of these wavelengths, the number of oligonucleotide molecules per antibody is estimated.

(12f) Conjugation of 5'-Trityl(S)-I-Q₁-I-3'-NH₂ to Antibody-Maleimide

A sample (10 nmoles) of 5'-Trityl(S)-I-Q₁-I-3'-NH₂, synthesized according to Example 2c, was diluted into PBS and a solution of silver nitrate in water was added to a final concentration of 85 mM. After vortexing the reaction was allowed to proceed at room temperature for 30 minutes. A precipitate formed which was centrifuged to the bottom of the tube. The clear supernatant, which contains the oligonucleotide with a 5' terminal thiol group and without the 5' trityl group (HS-5'-I-Q₁-I-3'-NH₂) was kept at 4°C until use.

A 6 nmole sample of ING-1-Maleimide (prepared essentially according to Example 10a) in PBS was reacted with 40 nmoles of HS-5'-I-Q₁-I-3'-NH₂ at 4°C for 16 hours. The reactants were diluted with PBS and eluted in 4 mL from a pre-washed Econopac 106-DG column (BioRad) to afford ING-1-Maleimide-S-5'-I-Q₁-I-3'Teg. The product was concentrated in a Centricon-300 concentration device by centrifugation at 1000g for 25 minutes. The sample was resuspended in fresh PBS and concentration by centrifugation was repeated a further 3 times until the ratio of optical densities at 260 nm and 280 nm was constant.

The optical density of these samples were examined in a spectrophotometer at 260 nm and 280 nm. The ratio

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of optical densities at these two wavelengths was calculated and, by using known extinction coefficients for the antibody and for the oligonucleotide (approximate molecular weight=16500) at each of these wavelengths, the number of oligonucleotide molecules per antibody was estimated.

Example 13

10 (13a) Annealing of cI to ING-1-I-Q₁-I

A sample of ING-1-Maleimide-S-(CH₂)₃-C(NH₂')NH-3'I-Q₁-I 5'Teg (24 pmoles), prepared according to Example 12a, and a sample of ING-1-Maleimide-S-(CH₂)₃-C(NH₂')NH-5'I-Q₁-I 3'Teg (25 pmoles) prepared according to Example 12d, were mixed in separate cuvettes with 16-fold excess of cI prepared according to Example 3c, in 50 mM PBS containing 1.0 mM EDTA and 100 mM NaCl, pH 7.2 at room temperature. The cuvettes were cooled to 20°C, loaded into a Cary 13 instrument, and analysed by UV light (260 nm) while the cuvette temperature was ramped up from 20°C to 80°C and then back down to 20°C at a rate of 0.5°C/min. Analysis of the data revealed that cI was able to hybridize to both the 3' and the 5' conjugates with ternary complex T_m of 61.5°C and 60.4°C respectively. Similar experiments using TMT-cI-TMT (Example 8) demonstrated the annealing of TMT-cI-TMT to ING-1-Maleimide-S-(CH₂)₃-C(NH₂')NH-3'I-Q₁-I 5'Teg.

30 (13b) Hybridization of ING-1-I-Q₁-I to ³²P labeled cI or ³²P labeled I in phosphate buffer and human serum

An ING-1-Maleimide-S-(CH₂)₃-C(NH₂')NH-3'I-Q₁-I 5'Teg (4ul; 1mg antibody/ml) solution (from Example 12a) and

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50 μ L 32 P-labeled cI solution (Example 4) were mixed with freshly prepared human serum (200 μ L) or PBS (200 μ L; pH7.2) and incubated at 37°C for 2 hours. Aliquots were then subjected to SDS PAGE electrophoresis on an 8-16% gel. The gels were examined by both autoradiography and on a phosphoimager system to show that 32 P-labeled cI was able to hybridize with ING-1-I-Q₁-I in human serum as well as phosphate buffer solution (pH 7.2). Samples were left at room temperature for 14 days in PBS and in serum and run on 8-16% SDS-PAGE gave similar patterns to each other and to gels incubated at 37°C for 2 hours suggesting that the conjugates were stable in serum for up to two weeks. 32 P-labeled I (Example 4) failed to show hybridization to ING-1-I-Q₁-I at any time as expected.

(13c) Hybridization of 90 Y-TMT-cI-TMT- 90 Y to ING-1-I-Q₁-I

An ING-1-Maleimide-S-(CH₂)₃-C(NH₂⁺)NH-3'I-Q₁-I-5'Teg (4ul; 1mg/ml) solution (from Example 12a) and 50 μ L 90 Y-TMT-cI-TMT- 90 Y (Example 9) were mixed in PBS (200 ul; pH7.2) and incubated at 37°C for 60 minutes. Aliquots of these mixtures were then subjected to SDS PAGE electrophoresis on an 8-16% gel. The gels were autoradiographed on a phosphoimager system to show that 90 Y-TMT-cI-TMT- 90 Y was able to hybridize with ING-1-I-Q₁-I.

(13d) Western blot to detect TMT on TMT-cI-TMT hybridized with ING1-I-Q₁-I.

Samples of I-Q₁-I (Example 2a) or ING-1-Maleimide-3'S-(CH₂)₃-C(=NH₂⁺)NH-I-Q₁-I-5'Teg (Example 12a) were mixed with the oligonucleotide TMT conjugate (TMT-cI-

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TMT; Example 8) on ice for 10 min. Aliquots of these reaction mixtures were mixed with SDS buffer and loaded onto two duplicate 8-16% polyacrylamide gels. The gels were subjected to electrophoresis at a constant voltage for 2 hours. One gel was electroblotted onto nitrocellulose paper using CAPS buffer for 20 minutes according to the manufacturer's protocol (Hoefer semi-dry transfer method). After washing thrice with a solution of 0.05% Tween 20 in PBS, the gel was blocked with a solution of 3% BSA in PBS at room temperature for 1 hour. Following further washing with Tween/PBS the gel was overlaid with a solution of a murine anti-TMT antibody (10 μ g/ml in PBS/Tween) and left overnight at room temperature. The western blot was developed using a goat anti-mouse IgG antibody conjugated to horseradish peroxidase (BioRad Western blot kit) and peroxidase substrate. The blot demonstrated that the TMTs of TMT-cI-TMT could be detected hybridized to bands that contained either I-Q₁-I or ING-1-I-Q₁-I.

The second gel was stained with ethidium bromide (5mg/ml in distilled water). Examination of the stained gel under UV light again demonstrated hybridization.

Example 14

Preparation of Ab-TMT by direct conjugation (ING-1/TMT)

TMT-NCS or a suitable derivative thereof can be conjugated to an antibody molecule to yield an antibody-TMT conjugate molecule that displays the ability to bind to a target antigen recognized by the antibody variable region. Such a conjugate molecule can be used to deliver metal ions that are chelated by the TMT moiety in order to localize and/or treat the lesion that is targeted by such an immunoconjugate. In one preferred

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embodiment, the antibody is selected such that it has a broad reactivity with an antigen molecule expressed on tumor cells, thereby providing an antibody-TMT conjugate that can deliver metals to the tumors for therapeutic or diagnostic purposes. The chimeric antibody, ING-1, (International patent publication WO 90/02569) consists of a murine variable region and a human immunoglobulin constant region. The antibody is produced by culturing a mouse myeloma cell line expressing the chimeric antibody essentially as described in the above-referenced publication. After purification, ING-1 is used at a concentration of 5.0 mg/mL in 50 mM sodium acetate and 150 mM sodium chloride buffer pH 5.6.

The conjugation of ING-1 to TMT-NCS is achieved by first adding 1.0M carbonate, 150 mM sodium chloride buffer, pH 9.3 to ING-1 until the antibody solution reaches a pH of 9.0. A sample of that ING-1 solution, containing 5 mg of protein, is then pipetted into an acid washed, conical, glass reaction vial. A solution of TMT-NCS is prepared by dissolving 100 mg in 10 mL of 1.0 M carbonate, 150 mM sodium chloride buffer, pH9.0. The conjugation reaction is started by the addition of 96.5 μ L of the TMT-NCS solution to the antibody to give a 4-fold molar excess of TMT-NCS over ING-1. The solution is stirred briefly to mix the reactants and then left in the dark at room temperature. After 16 hours, the ING-1/TMT conjugate is separated from unconjugated TMT by applying the reaction mixture to a PD10 chromatography column which has been pre-washed and equilibrated with 50 mM sodium acetate in 150 mM sodium chloride buffer, pH 5.6. The pure conjugate is eluted off the column with 2.5 mL of that same buffer.

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Example 15

Analytical tests on the ING-1/TMT conjugates.5 (15a) Analysis of Chelator to Antibody Ratio

10 The protein concentrations of ING-1 in the conjugate solutions were determined by the BioRad protein assay using bovine immunoglobulin as the protein standard.

15 In order to calculate the number of functional TMT molecules per antibody, ING-1/TMT is reacted with a solution of Europium chloride until saturation of the metal-binding capacity of the TMT occurs. A 0.375 mg aliquot of the ING-1/TMT in 2.5 ml in 0.05 M Tris HCl buffer pH 7.5 is pipetted into a 5 ml quartz cuvette. A 20 μ M Europium chloride (Europium chloride hexahydrate: Aldrich) solution in 0.05 M Tris HCl buffer pH 7.5 is prepared. An aliquot (50 μ L) of this Europium chloride solution is added to the cuvette containing ING-1/TMT and the resulting solution slowly stirred on a magnetic stirrer at room temperature for 10 min using a small magnetic stir bar placed in the cuvette. The fluorescence of the metal-ING-1/TMT complex is determined in a Perkin Elmer LS 50 spectrofluorometer using an excitation wavelength of 340 nm (10 nm slit width). The fluorescent emission is monitored at 618 nm using a 10 nm slit width and a 430 nm cutoff filter. The above procedure is repeated and fluorescent readings are made after each addition. Aliquots of Europium chloride are added until the increase in fluorescence intensity is less than 5% of the preceding reading. A dilution correction is applied to the fluorescence intensity measured at each mole ratio, to compensate for the change in volume of the test solution. Since each

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chelating site on the ING-1/TMT conjugate binds one Europium ion, and since Europium ion has to be in a chelate site for fluorescence to occur, this method allows the number of functional chelation sites to be quantitated.

Using this method, the ratio of TMT molecules per molecule of antibody is in the range 1: 1 to 2:1.

(15b) ING-1/TMT Immunoreactivity assay by ELISA

The antigen to which the antibody, ING-1, binds is prepared from LS174T or HT 29 cells (available from American Type Tissue Collection, ATTC) by scraping confluent monolayers of cells from the walls of culture flasks with a cell scraper. The cells from many flasks are combined and a sample is taken and counted to estimate the total number of cells harvested. At all times the cells are kept on ice. Following centrifugation of the cells at 1500 rpm for 10 minutes at 4°C, the cells are washed once in 25 mL ice-cold 50mM sodium phosphate buffer, pH 7.4 supplemented with 150 mM sodium chloride (PBS), pelleted under the same conditions and transferred in 10 mL PBS to an ice-cold glass mortar. The cells are homogenized at 4°C using a motor-driven pestle and then centrifuged at 3000 x g for 5 minutes. The antigen-rich supernatant is removed from the other cell debris and subjected to further centrifugation at 100,000 x g for one hour at 4°C. The pellet (antigen fraction) from this final step is suspended in 100 µL of PBS for every million cells harvested. Following an estimate of the protein concentration (BioRad BCA protein assay using bovine immunoglobulin as the protein standard) the antigen is stored at -20°C until use.

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Each well of a 96-well Costar microtiter plates is coated with antigen by adding 100 μ L/well of cell lysate (10 μ g/ml) prepared as above. The microtiter plates are allowed to dry overnight in a 37°C incubator. After washing the plate five times with 0.05% Tween-20 (Sigma) they are blotted dry. The wells of each plate are blocked by adding 125 μ L/well of a 1% BSA (bovine serum albumin, Sigma A-7906) solution in PBS and incubated for 1 hour at room temperature. The plates are washed five times with 0.05% Tween-20. Samples (50 μ L/well in duplicate) of ING-1/TMT conjugates and standard ING-1 antibody solutions are prepared at a range of concentrations in 1% BSA in PBS. Biotinylated ING-1 (1.0 μ g/mL in 0.1% BSA) is added to each well (50 μ L/well) and the plates are then incubated for 2 hours at room temperature. Following five washes with 0.05% Tween-20, the plates are blotted dry and incubated at room temperature for one hour with dilute (1:2000 in 0.1% BSA) streptavidin-alkaline phosphatase (Tago; #6567). After a further five washes, color is developed in each well upon the addition of 100 μ L per well of phosphatase substrate reagent (Sigma). After one hour at room temperature, the color is read using a 405 nm filter in a Titertek Multiscan microplate reader.

When tested by this procedure, the immunoconjugates of ING-1 with TMT are found to have immunoreactivity comparable to native ING-1.

(15c) ING-1/TMT Immunoreactivity Assay by Flow Cytometry

Target HT29 cells are grown to confluency in tissue culture flasks using McCoy's media supplemented with 10% fetal calf serum. The cells are harvested by scraping the flask walls with a cell scraper. Cells from

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many separate flasks are pooled, centrifuged to a pellet, resuspended at 5×10^5 /mL in a solution of ice-cold 50mM sodium phosphate with 150 mM sodium chloride buffer pH 7.4 (PBS) supplemented with 0.1% bovine serum albumin (Sigma) and 0.02% sodium azide (Flow buffer). The cells are washed in this same buffer and then counted. An antibody standard curve is constructed by diluting ING-1 with an irrelevant (non binding), isotype-matched control antibody (human IgG₁) to give a number of samples ranging in ING-1 content from 10% to 100%. The standard curve is made in flow buffer so that each sample contains 1.0 μ g protein per mL. Samples from the standard curve and unknowns are then incubated with 5×10^5 HT29 cells at 4°C for 1 hour. After extensive washing to remove unbound antibody, the cells are resuspended in 100 μ L flow buffer and incubated at 4°C for 1 hour with goat-anti-human antibody labeled with fluoresceine isothiocyanate (FITC). After further washing in flow buffer the samples are analyzed by flow cytometry on a Coulter EPICS 753 flow cytometer. Fluoresceine FITC and propidium iodide (PI) are excited using the 488 nm emission line of an argon laser. The output is set at 500 mw in light regulation mode. Single cells are identified by 90 degree and forward angle light scatter. Analysis windows are applied to these parameters to separate single cells from aggregates and cell debris. Fluorescence from FITC and propidium are separated with a 550nm long pass dichroic filter and collected through a 530 nm band pass filter (for FITC), and a 635 nm band pass filter (for PI). Light scatter parameters are collected as integrated pulses and fluorescence is collected as log integrated pulses. Dead cells are excluded from the assay by placing an analysis window on cells negative for PI uptake. The mean fluorescence per sample (weighted average from 2500

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cells) is calculated for each histogram. FITC calibration beads are analysed in each experiment to establish a standard curve. The average fluorescence intensity for each sample is then expressed as the average FITC equivalents per cell. Immunoreactivity is calculated by comparing the average fluorescence intensity of the unknown sample with values from the standard curve. Samples of ING-1/TMT have immunoreactivity values comparable to the native ING-1 antibody by this method.

(15d) Determination of aggregate formation by size-exclusion HPLC.

A 30 cm x 7.5 mm TSK-G3000SW size-exclusion HPLC column (Supelco) fitted with a guard column of the same material is equilibrated with 12 column volumes of 10 mM sodium phosphate buffer pH 6.0 supplemented with 150 mM sodium chloride using a Waters 600E HPLC system with a flow rate of 1.0 mL per minute at 400-600 PSI. A sample (25 μ L) of BioRad gel filtration protein standards is injected on to the column. The retention time of each standard is monitored by a Waters 490 UV detector set at 280 nm. Following the recovery of the final standard from the column, it is washed with a further 10 volumes of 10 mM sodium phosphate buffer pH 6.0 supplemented with 150 mM sodium chloride. Samples (50 μ L) of either native ING-1 antibody or ING-1/TMT at 200 μ g/mL are injected on to the column and their retention times recorded. From the areas of the retained peaks and the retention time, the amount of aggregated material in the ING-1/TMT sample is calculated.

By this method the native ING-1 antibody has a retention time of 9.1 minutes. ING-1/TMT has a major peak also at 9.1 minutes but a minor peak, attributable

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to aggregates, can sometimes be seen at 7.3 minutes. By comparison of the peak areas, the aggregate peak is less than 5% of the total.

5 (15e) Radiolabeling of ING-1/TMT with ^{90}Y .

A volume of radioactive Yttrium chloride (^{90}Y in 0.04M hydrochloric acid at a specific activity of >500 Ci/mg: Amersham-Medipysics) is neutralized using two
10 volumes of 0.5 M sodium acetate pH 6.0. The neutralized ^{90}Y (1.0 mCi) is added to 1.0 mL of ING-1/TMT (1 mg/mL) in 50 mM sodium acetate buffer containing 150 mM sodium chloride at pH 5.6. The labelling is allowed to proceed for one hour and then the reaction mixture is loaded on
15 to a PD-10 chromatography column which has been pre-washed and equilibrated in a buffer containing 50mM sodium phosphate with 150 mM sodium chloride pH 7.4 (PBS). The sample is eluted from the column with 1.5 mL of PBS. Fractions of radiolabeled ING-1/TMT (0.5 mL)
20 are collected, assayed for radioactivity, and pooled. The labeling efficiency is determined by removing 1.0 μL of the sample and spotting it on to a Gelman ITLC-SG strip. The strip is developed in a glass beaker containing 0.1 M sodium citrate, pH 6.0 for a few
25 minutes until the solvent front has reached three-quarters of the way to the top of the paper. The strip is inserted into a System 200 Imaging Scanner (Bioscan) which has been optimized for ^{90}Y and is controlled by a Compaq 386/20e computer. In this system free ^{90}Y
30 migrates at the solvent front while the ING-1/TMT (^{90}Y) remains at the origin.

Using this system more than 98% of the total ^{90}Y radioactivity is always found associated with ING-1/TMT at the origin.

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(15f) Labeling ING-1/TMT with fluorescent metals.

Binding of lanthanides such as europium (3⁺) to chelating agents that contain an aromatic moiety held close to the co-ordination sphere can lead to "sensitized" fluorescence wherein light is absorbed through the aromatic system and the energy is transferred to the metal. The metal then produces emissions characterized by a very large Stokes shift and fluorescence lifetimes of up to several seconds. A 0.5 mg aliquot of the ING-1/TMT in 2.5 mL in 0.05 M Tris HCl buffer pH 7.5 was pipetted into a 4 mL conical reaction vial containing a small stirring bar. A 250 μ M europium chloride (europium chloride hexahydrate: Aldrich) solution in 0.05 M Tris HCl buffer pH 7.5 was prepared. An aliquot (50 μ L) of this europium chloride solution was added to the reaction vial containing ING-1/TMT, and the resulting solution was stirred very slowly on a magnetic stirrer at room temperature. The labelling was allowed to proceed for one hour and then the reaction mixture was loaded on to a PD-10 chromatography column which had been pre-washed and equilibrated in a buffer containing 10 mM sodium phosphate and 150 mM sodium chloride at pH 6.0 (PBS). The sample was eluted from the column with 3.5 mL of PBS. The fluorescence of a 50 μ L sample of the metal-ING-1/TMT complex was determined in a Perkin Elmer LS 50 spectrofluorometer using an excitation wavelength of 340 nm (10 nm slit width). The fluorescent emission was recorded at 618 nm using a 10 nm slit width and a 430 nm cutoff filter. Each functional chelating site on the ING-1/TMT conjugate binds one europium ion. Using this method, between 1 and 3 fluorescent europium ions were bound per molecule of antibody.

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Example 16

(16a) Flow Cytometry of binding of fluorescent cI to
cells treated with ING-1-I-Q₁-I.

5
ING-1-Maleimide-S-(CH₂)₃-C(NH₂⁺)NH-3'I-Q₁-I 5'Teg
was prepared as in Example 12a. ING-1-Maleimide-S-
(CH₂)₃-C(NH₂⁺)NH-5'I-Q₁-I 3'Teg was prepared as in Example
12b. Fluorescently labeled CY5.18-I-Q₁-I was prepared
10 as in Example 2d. Fluorescently labeled CY5.18-cI and
CY5.18-I were prepared as in Example 3e. Flow cytometry
was carried out essentially as described in 12c except
that the fluorescent dye CY5.18 was used in place of
FITC. HT-29 cells (0.5x10⁶) were incubated on ice for 30
15 min. with 1 µg each of the ING-1-I-Q₁-I samples. The
cells were washed x2 with flow buffer and pelleted at
1400 rpm x 5 minutes between washes. Next the cells in
each sample were incubated with 5 µg CY5.18-cI or
CY5.18-I for 3 hours on ice. Some cells were incubated
20 with CY5.18-cI and CY5.18-I alone. After extensive
washing with flow buffer, the cells were subjected to
analysis on a fluorescence activated cell sorter. CY5-
18 calibration beads were analysed to establish a
standard curve of relative fluorescence intensity versus
25 CY5-18 concentration. The mean fluorescence per sample
(weighted average from 2500 cells) was calculated for
each histogram. The average fluorescence intensity for
each sample was then expressed as the average CY5-18
equivalents per cell. Identical experiments were
30 carried out in which the medium used for incubation of
the cells with the components was 100% fetal calf serum
in place of flow buffer.

Preliminary experiments showed that the time taken
to maximize hybridization of CY5-18-cI to ING-1-
35 Maleimide-S-(CH₂)₃-C(NH₂⁺)NH-3'I-Q₁-I 5'Teg on the surface

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of HT-29 cells was about 3 hours. In Table 2 there was no difference between flow buffer and 100% FCS in the degree of hybridization in each preparation suggesting that the end-capped oligonucleotide strands were stable to nuclease digestion. The data suggest that large amounts of CY5.18-cI can hybridize to both conjugates bound to the surface of cells. There was relatively little non-specific binding to the cells either by the fluorescent oligonucleotides themselves or by hybridization of CY5-18-I-Q_I-I to the conjugates.

Table 2

	Additions to HT-29 Cells	Relative Fluorescence in PBS	Relative Fluorescence in Fetal Calf Serum
	None	<2000	<2000
	CY5.18-I-Q _I -I	<2000	<2000
	CY5.19-cI	<2000	<2000
	ING1-3'I-Q _I -I 5'Teg + CY5.18-I-Q _I -I	<2000	<2000
	ING1-3'I-Q _I -I 5'Teg + CY5.18-cI	31481	27407
	ING1-5'I-Q _I -I 3'Teg + CY5.18-I-Q _I -I	<2000	<2000
	ING1-5'I-Q _I -I 3'Teg + CY5.18-cI	25925	22222

(16b) Binding of ⁹⁰Y-TMT-cI-TMT-⁹⁰Y to HT-29 cells treated with ING-1-I-Q_I-I.

TMT-cI-TMT was radiolabeled to a specific activity of 0.1 μ Ci/pmole as described in Example 9. ING-1-TMT⁹⁰Y was prepared as in Example 15e. ING-1-Maleimide-S-(CH₂)₃-C(NH₂)NH-3'I-Q_I-I 5'Teg was prepared as in Example 12a. Three tubes each containing 1x10⁵ HT-29

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cells in DMEM medium supplemented with 10% fetal calf serum were prepared and kept at 4°C throughout. Using matched protein concentrations in all tubes to ensure that the amount of added antibody was equal, the tubes were treated as shown in Table 3 below.

Table 3

Time (min)	Sample 1	Sample 2	Sample 3
0	+ING-1-I-Q _I -I	0	0
60	Wash x2	Wash x2	Wash x2
90	+ cI-(TMT ⁹⁰ Y) ₂	ING-1-TMT ⁹⁰ Y	Prehybridized ING-1-I-Q _I -I with cI-(TMT ⁹⁰ Y) ₂
150	Wash x2	Wash x2	Wash x2
180	Centrifuge cells and count pellet radioactivity	Centrifuge cells and count pellet radioactivity	Centrifuge cells and count pellet radioactivity
mean DPM per 10 ⁵ cells	605	541	654

Both sample 1 and sample 3 show higher radioactivity associated with the cell pellet than does sample 2 (directly labeled ING-1-TMT⁹⁰Y) suggesting that a delivery system consisting of 2 separate components can deliver more radioactivity than a directly labeled antibody.

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(16c) Binding of Europium labeled ING-1/TMT and TMT-cI-TMT conjugates to HT-29 cells and HT-29 cells treated with ING-1-I-Q₁-I, respectively

5 The TMT-cI-TMT conjugate was labeled with Europium ions as described in Example 8a and ING-1/TMT was labeled with Europium ions as described in Example 15f. Standard curves were created for both conjugates in concentration ranges of 100 picomoles/100 μ l to 6
10 femtomoles/100 μ l. HT-29 cells were grown to confluence in McCoy's media containing 10% FCS and 50 μ g/ml of gentamycin. The cells were washed with phosphate buffered saline, and 5 ml of Trypsin Versene was added. The HT-29 cells were then incubated at 37°C in 5% CO₂,
15 for 15 minutes, complete media (5 ml) was added, and the cells were removed and washed in PBS. The HT-29 cells were then blocked with 10 μ g of sheared salmon sperm DNA per 10⁶ cells at 4°C for 30 minutes, washed in PBS and used in the hybridization assay as follows. 5 x 10⁵ HT-
20 29 cells were added to a 100 μ l working dilution of Ing-1/TMT or Ing-1-I-Q₁-I and the mixture was incubated for 30 minutes on ice. The cells were washed twice in 2 ml of a wash buffer (PBS + 0.1% BSA + 0.01% NaN₃) at 1400 RPM for 5 minutes. A working dilution of TMT-cI-TMT
25 (100 μ l) was then added to the appropriate tubes and the reaction mixture was incubated on ice for 3 hours followed by washing twice in 2 ml of wash buffer (PBS + 0.1% BSA + 0.01% NaN₃) at 1400 RPM for 5 minutes.

Each binding study was done in triplicate. The
30 cell suspensions after binding and hybridization as above were kept at 4°C in test tubes until use (10 minutes to one hour). The Europium fluorescence was measured in a Delfia 1232 time-resolved fluorimeter by aliquoting four 100 μ l portions into separate wells in a
35 Costar EIA/RIA 96-well plate from each tube after

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vortexing. The results were processed as described in Example 15f. The counts per seconds (cps) of the Eu-TMTcI-TMT-Eu treated cells were considered as background and were subtracted from the cps data from the hybridization experiments (Eu-TMT-cI-TMT-Eu/Ing1-I-Q₁-I). This result and the cps data from the Ing-1/TMT-Eu binding experiment were translated as picomoles of bound TMT molecules per cell from the individual standard curves created independently. The results are described in Table 4 below.

The results are described as fluorescence counts per second (cps) or number (#) of picomoles per 10⁵ cells. ING-1/TMT-Eu and ING-conjugates: 0.25 µg or 1.65 picomoles; Eu-TMT-cI-TMT-Eu: 100 ng or 15 picomoles per 5 X10⁵ cells.

Table 4

Conjugate	Ratio TMT or DNA per Ab	CPS	# picomoles	% binding	#picomole s Eu-TMT- cI- TMT-Eu
ING-1/TMT-Eu	2.0	17828	0.099	27.3	0.198
ING-1-3'-I-Q ₁ I- 5'-TEG + Eu- TMT-cI-TMT-Eu	1.25	84613	0.400	48.5	0.80
ING-1-5'-I-Q ₁ I- 3'-TEG + Eu- TMT-cI-TMT-Eu	1.88	125857	0.633	51.0	1.27

The invention has been described in detail with particular reference to certain preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

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SEQUENCE LISTING

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Shearman, Clyde W.
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Sen, Arup
Black, Christopher D. V.
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OLIGONUCLEOTIDE CONJUGATES OF CHELATED RADIONUCLIDES
- (iii) NUMBER OF SEQUENCES: 36
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- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTATGGACGG AG

12

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTATGGACGG AGA

13

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTATGGACGG AGAA

14

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTATGGACGG AGAAG

15

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTATGGACGG AGAAGC

16

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTATGGACGG AGAAGCT

17

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTATGGACGG AGAAGCTA

18

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTATGGACGG AGAAGCTAA

19

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGAGAAGCT AA

12

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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-89-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACGGAGAAGC TAA

13

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACGGAGAAG CTAA

14

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGACGGAGAA GCTAA

15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGGACGGAGA AGCTAA

16

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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ATGGACGGAG AAGCTAA

17

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TATGGACGGA GAAGCTAA

18

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTAGCTTCTC CG

12

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTAGCTTCTC CGT

13

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTAGCTTCTC CGTC

14

(2) INFORMATION FOR SEQ ID NO:19:

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-91-

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTAGCTTCTC CGTCC

15

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTAGCTTCTC CGTCCA

16

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTAGCTTCTC CGTCCAT

17

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTAGCTTCTC CGTCCATA

18

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTAGCTTCTC CGTCCATAA

19

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCCGTCCAT AA

12

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTCCGTCCA TAA

13

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTCTCCGTCC ATAA

14

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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-93-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTTCTCCGTC CATAA

15

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCTTCTCCGT CCATAA

16

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AGCTTCTCOG TCCATAA

17

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TAGCTTCTCC GTCCATAA

18

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "N equals Teg-T."

- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 50
 (D) OTHER INFORMATION: /note= "N equals NH2-T."

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

NCTTATGGAC GGAGAAGCTA AACTCTCTTA TGGACGGAGA AGCTAATCTN

50

- (2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCTTATGGAC GGATCOGCTA ATCTTATGGA CGGATCOGCT AATCT

45

- (2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 52 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "N equals NH2 or Teg."

- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 51
 (D) OTHER INFORMATION: /note= "N equals NH2 or Teg."

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

NTCTTATGGA CGGAGAAGCT AACTCTCTT ATGGACGGAG AAGCTAATCT NT

52

- (2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

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(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "N equals NH2-T."

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 50
 (D) OTHER INFORMATION: /note= "N equals Teg-T."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

NCTTATGGAC GGAGAAGCTA AACTCTCTTA TGGACGGAGA AGCTAATCTN

50

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "N equals NH2-T."

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 20
 (D) OTHER INFORMATION: /note= "N equals NH2-T."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

NTAGCTTCTC CGTCCATAAN

20

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "N equals NH2-T."

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 20
 (D) OTHER INFORMATION: /note= "N equals NH2-T."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

NTATGGAGCG AGAAGCTAAN

20

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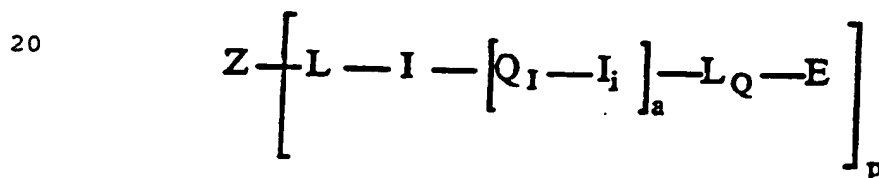
We claim:

1. A non-radioactive targeting immunoreagent that comprises a tumor antigen recognizing moiety, one or more oligonucleotides comprised of non self-associating oligonucleotide sequences, and one or more linking groups.

2. A radioactive targeting immunoreagent that comprises an oligonucleotide comprised of an oligonucleotide sequence that is complementary in sequence to and capable of hybridization with one or more fragments of a non-self-associating oligonucleotide sequence, one or more chelating agents, one or more linking groups and one or more radionuclides.

3. A targeting immunoreagent that comprises moieties represented by the structure 1:

Structure 1



wherein:

Z comprises the residue of an immunoreactive protein;

L and L_Q are independently a chemical bond or a linking group;

I comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous contiguous sequences, the individual homologs of said family being comprised of from 12 to about 30 nucleotide units, provided that contiguous sequences of six or more

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nucleotide units of said oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous nucleotide units anywhere in structure 1;

5 Q_1 is a spacing group;

a is 0 to about 6;

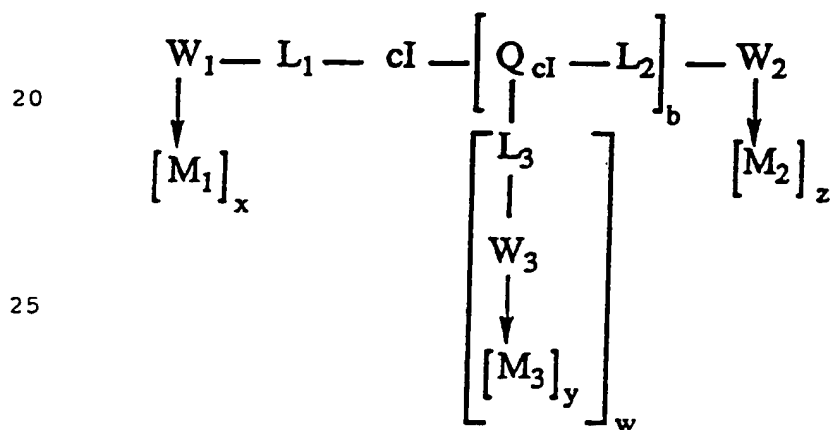
I_1 comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units, a contiguous sequence therein comprising a
10 portion of I ;

E is an end capping group; and

p is an integer from 1 to about 10.

4. A radioactive targeting reagent comprised of
15 moieties represented in the structure 2:

Structure 2



30 wherein:

cI comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units wherein said contiguous sequence contains therein one or more members of a family of homologous contiguous
35 sequences, the individual homologs of said family being

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comprised of from 12 to about 30 nucleotide units; the nucleotide sequences of said members of said family of homologous contiguous sequences are complementary to the nucleotide sequences of members of the set of
5 oligonucleotides in a coadministerable targeting immunoreagent; and contiguous sequences of six or more nucleotide units of said complementary oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous nucleotide units anywhere in
10 structure 2;

Q_{ci} is a spacing group;

L_1 , L_2 , and L_3 are independently a chemical bond or a linking group;

W_1 , W_2 , and W_3 are residues of chelating groups;

15 M_1 , M_2 and M_3 are comprised of elements with oxidation states equal to or greater than +1, at least one of which is a radionuclide;

x , y , and z are independently zero or one provided that at least one of x , y , or z is one; and

20 w and b are independently zero or an integer from 1 to about 4.

5. The reagent of claim 3 wherein a is an integer from one to about 6.

25

6. The reagent of claim 3 wherein Z is the residue of an antibody or antibody fragment.

7. The reagent of claim 6 wherein the antibody is
30 selected from ING-1; B72.3; 9.2.27; D612; UJ13A; NRLU-10; 7E11C5; CC49; TNT; PR1A3; B174; B43; and anti-HLB antibodies.

8. The reagent of claim 3 wherein L or L_c is a
35 residue of a heterobifunctional cross-linking reagent.

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9. The reagent of claim 8 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-Iminothiolane, and N-succinimidyl S-acetylthioacetate.

10. The reagent of claim 3 wherein L or L₂ is a residue of a modified nucleotide moiety comprising a reactive functional group.

11. The reagent of claim 10 where the reactive functional group is selected from the group consisting of amine groups and sulfhydryl groups.

12. The reagent of claim 3 wherein I and I₁ are DNA.

13. The reagent of claim 3 wherein I has the sequence shown in SEQ ID NO: 8.

14. The reagent of claim 3 wherein Q₁ is the residue of an oligonucleotide.

15. The reagent of claim 14 wherein the oligonucleotide has the sequence ACTCTC.

16. The reagent of claim 3 wherein E is the residue of a modified nucleotide group resistant to exonuclease activity.

17. The reagent of claim 16 wherein the modified nucleotide comprises a poly(alkylene glycol) phosphate diester.

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18. The reagent of claim 17 wherein the diester is tetra(ethylene glycol) phosphate diester.

5 19. The reagent of claim 4 wherein CI has the sequence show in SEQ ID NO: 23.

20. The reagent of claim 4 wherein Q_i is the residue of an oligonucleotide.

10 21. The reagent of claim 20 wherein the oligonucleotide has the sequence ACTCTC.

15 22. The reagent of claim 4 wherein L₁, L₂ or L₃ is the residue of a heterobifunctional cross-linking reagent.

20 23. The reagent of claim 22 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-Iminothiolane, and N-succinimidyl S-acetylthioacetate.

25 24. The reagent of claim 4 wherein L₁, L₂ or L₃ is the residue of a modified nucleotide moiety containing a reactive functional group.

30 25. The reagent of claim 24 wherein the reactive functional group is selected from the group consisting of amine groups and sulfhydryl groups.

35 26. The reagent of claim 4 wherein W₁, W₂ or W₃ independently contains a polycarboxylic acid group.

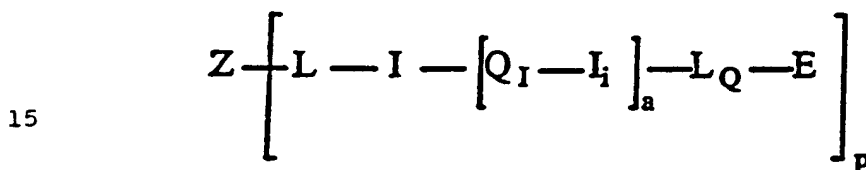
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27. The reagent of claim 4 wherein W_1 , W_2 or W_3 is independently selected from the group consisting of B4A, P4A, TMT, DCDTPA, PheMT, macroPheMT, and macroTMT.

5 28. The reagent of claim 4 wherein the radionuclide is selected from ^{212}Pb , ^{212}Bi , ^{90}Y , ^{177}Lu , ^{186}Re , ^{188}Re , ^{64}Cu , $^{99\text{m}}\text{Tc}$, ^{111}In , and ^{87}Y .

10 29. A method of making a compound of the structure:
Structure 1



wherein:

20 Z comprises the residue of an immunoreactive protein;

L and L_Q are independently a chemical bond or a linking group;

25 I comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units wherein said contiguous sequence contains one or more members of a family of homologous contiguous sequences, the individual homologs of said family being comprised of from 12 to about 30 nucleotide units, provided that contiguous sequences of six or more
30 nucleotide units of said oligonucleotide do not hybridize with any other contiguous sequences of six or more contiguous nucleotide units anywhere in structure 1;

Q_1 is a spacing group;

35 a is an integer from 1 to about 6;

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I_1 comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units, a contiguous sequence therein comprising a portion of I;

5 E is an end capping group; and

p is an integer from 1 to about 10;

comprising:

- (i) derivatizing Z with a precursor to a residue of L under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $Z-[L]_p$;
- (ii) derivatizing a precursor to a residue of E with a precursor to a residue of L_0 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L_0-E ;
- (iii) derivatizing a precursor to a residue of L_0-E with a precursor to a residue of I_1 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of I_1-L_0-E ;
- (iv) derivatizing a precursor to a residue of I_1-L_0-E with a precursor to a residue of Q_1 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $Q_1-I_1-L_0-E$;
- (v) derivatizing a precursor to a residue of $Q_1-I_1-L_0-E$ with a precursor to a residue of I_1 and then with a precursor to a residue of Q_1 under conditions and for a time period sufficient to form a covalent complex which is a precursor of a residue of $[Q_1-I_1]_a-L_0-E$;
- (vi) derivatizing a precursor to a residue of $[Q_1-I_1]_a-L_0-E$ with a precursor to a residue of I under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $I-[Q_1-I_1]_a-L_0-E$;
- (vii) derivatizing a precursor to a residue of $I-[Q_1-I_1]_a-L_0-E$ with a precursor to a residue of L under

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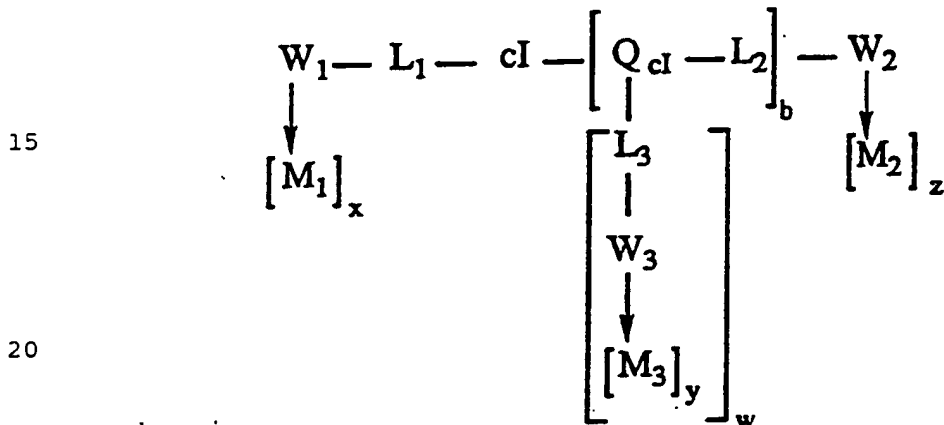
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conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L-I-[Q₁-I₁]_a-L₀-E; and

- (viii) derivatizing a precursor to a residue of L-I-[Q₁-I₁]_a-L₀-E with a precursor to a residue of Z-[L]_p under conditions and for a time period sufficient to form a covalent complex Z-[L-I-[Q₁-I₁]_a-L₀-E]_p.

30. A method of making a compound of the structure:

Structure 2



wherein:

cI comprises an oligonucleotide comprised of a contiguous sequence of from 12 to about 50 nucleotide units wherein said contiguous sequence contains therein one or more members of a family of homologous contiguous sequences, the individual homologs of said family being comprised of from 12 to about 30 nucleotide units; where the nucleotide sequences of said members of said family of homologous contiguous sequences are complementary to the nucleotide sequences of members of the set of oligonucleotides in a coadministerable targeting immunoreagent; and contiguous sequences of six or more nucleotide units of said complementary oligonucleotide do not hybridize with any other contiguous sequences of

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six or more contiguous nucleotide units anywhere in structure 2;

Q_{CI} is a spacing group;

L_1 , L_2 , and L_3 are independently a chemical bond or a linking group;

W_1 , W_2 , and W_3 are residues of chelating groups;

M_1 , M_2 , and M_3 are comprised of elements with oxidation states greater than +1, at least one of which is a radionuclide;

x , y , and z are independently zero or one provided that at least one of x , y , or z is one; and

w and b are zero or an integer from 1 to about 4; comprising:

(i) derivatizing a precursor to a residue of L_1 with a precursor to a residue of CI under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L_1-CI ;

(ii) derivatizing a precursor to a residue of Q_{CI} with a precursor to a residue of L_3 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $Q_{CI}-[L_3]_w$;

(iii) derivatizing a precursor to a residue of L_1-CI with a precursor to a residue of $Q_{CI}-[L_3]_w$ under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $L_1-CI-(Q_{CI}-[L_3]_w)$;

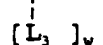
(iv) derivatizing a precursor to a residue of $L_1-CI-(Q_{CI}-[L_3]_w)$ with a precursor to a residue of L_2 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of $L_1-CI-(Q_{CI}-[L_3]_w-L_2)$;

(v) derivatizing a precursor to a residue of $L_1-CI-(Q_{CI}-[L_3]_w-L_2)$ with a precursor to a residue of $Q_{CI}-[L_3]_w$ and then with a precursor to a residue of L_2 under conditions and for a time period sufficient to form a

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covalent complex which is a precursor to a residue of
 $L_1\text{-Cl-}[\text{QcI-L}_2]_b;$



5

(vi) derivatizing a precursor to a residue of
 $L_1\text{-Cl-}[\text{QcI-L}_2]_b$



10

with a precursor to a residue of W_1 , W_2 , and W_3 under
 conditions and for a time period sufficient to form a
 covalent complex which is a precursor to a residue of
 $W_1\text{-L}_1\text{-Cl-}[\text{QcI-L}_2]_b\text{-W}_2;$

15



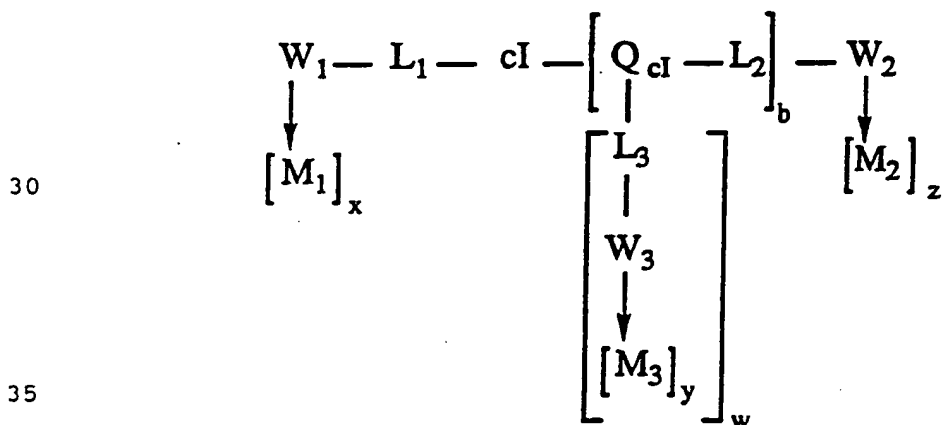
(vii) derivatizing a precursor to a residue of
 $W_1\text{-L}_1\text{-Cl-}[\text{QcI-L}_2]_b\text{-W}_2$

20



with a precursor to a residue of $[M_1]_x$, $[M_2]_z$, and $[M_3]_y$
 under conditions and for a time period sufficient to
 form a complex

25



30

35

31. The method of claim 29 wherein Z is an
 antibody or antibody fragment.

40

32. The antibody of claim 31 wherein the antibody
 is selected from ING-1; B72.3; 9.2.27; D612; UJ13A;

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NRLU-10; 7E11C5; CC49; TNT; PR1A3; B174; B43; and anti-HLB antibodies.

5 33. The method of claim 29 wherein L or L₀ is a residue of a heterobifunctional cross-linking reagent.

10 34. The method of claim 33 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-Iminothiolane, and N-succinimidyl S-acetylthioacetate.

15 35. The method of claim 29 wherein L or L₀ is a residue of a modified nucleotide moiety containing a reactive functional group.

20 36. The method of claim 35 wherein reactive functional group is selected from the group consisting of amine groups and sulfhydryl groups.

25 37. The method of claim 29 wherein I and I₁ are DNA.

38. The method of claim 29 wherein I has the sequence shown in SEQ ID NO: 8.

30 39. The method of claim 29 wherein Q₁ is a residue of an oligonucleotide.

40. The method of claim 39 wherein the oligonucleotide has the sequence ACTCTC.

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41. The method of claim 29 wherein E is a residue of a modified nucleotide resistant to exonuclease activity.

5 42. The method of claim 41 wherein the modified nucleotide comprises a poly(alkylene glycol) phosphate diester.

43. The method of claim 42 wherein the phosphate
10 diester is tetra(ethylene glycol) phosphate diester.

44. The method of claim 30 wherein cI has the sequence show in SEQ ID NO: 23.

15 45. The method of claim 29 wherein Q_i is a residue of an oligonucleotide.

46. The method of claim 45 wherein the
20 oligonucleotide has the sequence ACTCTC.

47. The method of claim 30 wherein L₁, L₂ or L₃ is a residue of a heterobifunctional cross-linking reagent.

48. The method of claim 47 wherein the
25 heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-
30 Iminothiolane, and N-succinimidyl S-acetylthioacetate.

49. The method of claim 30 wherein L₁, L₂ or L₃ is a residue of a modified nucleotide moiety containing a reactive functional group.

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50. The method of claim 49 wherein the reactive functional group is selected from the group consisting of amine groups and sulfhydryl groups.

5 51. The method of claim 30 wherein W_1 , W_2 or W_3 contains a polycarboxylic acid group.

52. The method of claim 30 wherein W_1 , W_2 or W_3 is selected from the group consisting of residues of B4A,
10 P4A, TMT, DCDTPA, PheMT, macroPheMT, and macroTMT.

53. The method of claim 30 wherein one of M_1 , M_2 and M_3 is a radioactive metal ion isotope.

15 54. The method of claim 53 wherein the radioactive metal ion isotope is ^{90}Y .

55. A pharmaceutical composition comprising a compound of claim 3 dissolved or dispersed in a
20 pharmaceutically acceptable carrier.

56. A pharmaceutical composition comprising a compound of claim 4 dissolved or dispersed in a pharmaceutically acceptable medium.

25 57. A method of treating a tumor in a mammal comprising administering to said mammal an effective dose of a non-radioactive targeting immunoreagent of claim 3 in a pharmaceutically acceptable medium, waiting
30 for a time period sufficient for said non-radioactive targeting immunoreagent to accumulate at the tumor site in said mammal, and subsequently, administering an effective dose of a radioactive targeting reagent of claim 4 in a pharmaceutically acceptable medium to said
35 mammal, waiting for a time period sufficient for said

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radioactive targeting reagent to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said tumor site in said mammal.

5

58. A method of diagnostic imaging in a mammal comprising administering to said mammal an imaging effective dose of a non-radioactive targeting immunoreagent of claim 3 in a pharmaceutically acceptable medium, waiting for a time period sufficient for said non-radioactive targeting immunoreagent to accumulate at the imaging site in said mammal, and subsequently, administering an imaging effective dose of a radioactive targeting reagent of claim 4 in a pharmaceutically acceptable medium to said mammal, waiting for a time period sufficient for said radioactive targeting reagent to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said imaging site in said mammal and generating an image of said mammal.

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59. The reagent of claim 3 wherein I is a double helix.

25

60. The reagent of claim 4 wherein cI is a double helix.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11637

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9, 85.8; 514/44, 2; 530/391.1, 391.3, 391.5, 391.7, 391.9, 345, 402; 536/22.1, 24.2; 558/17; 600/1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0,490,434 (KUIJERS) 17 June 1992, see entire document.	1-60
Y	Nucleic Acids Research, Volume 16, issued March 1988, A. Oser et al., "Sensitive non-radioactive dot-blot hybridization using DNA probes labelled with chelate group substituted psoralen and quantitative detection by europium ion fluorescence", pages 1181-1196, see entire document.	2, 4, 19 - 28, 30, 44, 47-54, 56, 60
Y	US, A, 4,587,044 (MILLER et al.) 06 May 1986, see entire document.	1-60

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T* Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

27 February 1994

Date of mailing of the international search report

MAR 11 1994

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Washington, D.C. 20231

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Authorized officer

RON SCHWADRON

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

In. .tional application No.
PCT/US93/11637

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A61K 39/395, 49/02, 37/02, 31/70, 35/14; C07K 13/00, 15/00, 15/28, 1/00, 1/10; C07H 21/04, 21/02; C07C 331/00;
A61M 36/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/9, 85.8; 514/44, 2; 530/391.1, 391.3, 391.5, 391.7, 391.9, 345, 402; 536/22.1, 24.2; 558/17; 600/1

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, BIOSIS, CHEM ABS, DERWENT WPI, EMBASE, APS, search terms: author names, radiolabel, oligonucleotide, radionucleotide, chelate, antibody, protein, peptide, conjugate, immunoconjugate, nucleotide, DNA, RNA, tumor, targeting